Population biology of Ixodes ticks

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Award date:
2011

Awarding institution:
University of Bath

Population biology of *Ixodes* ticks

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

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September 2011

"Science is the knowledge of consequences, and dependence of one fact upon another."

— Thomas Hobbes, *Leviathan.*

"'Is it about a bicycle?', he asked."

— Flann O'Brien, *The third policeman.*

Acknowledgements

First and foremost I would like to thank my late supervisor Klaus Kurtenbach, whose knowledge and enthusiasm about ticks, and about sharks, was truly infectious, and who is still dearly missed.

This work would not have been possible to complete without the outstanding support that I received from Dr Gabi Margos and Professor Stuart Reynolds. They guided me through my PhD project as the best supervisors one could ask for.

I am also most grateful to Dr Ed Feil, who made crucial suggestions about my work on tick endosymbionts, and who put up with my ignorance in phylogenetic matters.

My friends and colleagues Stephanie Vollmer and Ruth Dinnis shared many hours of tick collecting, tick crushing, and tick sequence editing, which made these tasks far more entertaining than if I had been on my own.

Dr Richard Birtles and Dr Kevin Bown were immensely helpful in establishing the blood meal analysis scheme in our lab, and provided ample suggestions and materials.

Dr Nick Waterfield kindly agreed to let me use his clean room facilities for my experiments on borrelia.

Stavros Gavkalias performed most of the PCR screening and amplification of *Midichloria* samples, which contributed greatly to the chapter on tick endosymbionts.

Several members of the tick research community made very useful suggestions and offered kind words of encouragement, especially Sarah Randolph, Jean Tsao, Joe Piesman, and Reuben Kaufman.

The members of my lab are to be thanked for giving helpful advice, having lunch breaks by the lake, and sharing my belief in a life outside the laboratory.

My wonderful family encouraged and supported me through my entire studies in every possible way.

Finally, I would like to thank Sara for being my North, South, East, and West whenever I lost my compass.

Publications

- **Host migration impacts on the phylogeography of Lyme Borreliosis spirochaete species in Europe.** (2011). Stephanie A. Vollmer, Antra Bormane, Ruth E. Dinnis, **Frederik Seelig**, Andrew D.M. Dobson, David M. Aanensen, Marianne C. James, Michael Donaghy, Sarah E. Randolph, Edward J. Feil, Klaus Kurtenbach, Gabriele Margos. *Environmental Microbiology.* 13(**1**) 184-192.

- **Analysing the Genetic Diversity of *Ixodes ricinus* ticks using Multilocus Sequence Typing.** Ruth E. Dinnis, **Frederik Seelig**, Antra Bormane, Michael Donaghy, Stephanie A. Vollmer, Edward J. Feil, Gabriele Margos, Klaus Kurtenbach. Submitted to *Ticks and tick-borne diseases. See Appendix E for a current version of the manuscript.*

Abstract

The hard tick *Ixodes ricinus* is one of the most important species of disease vectors worldwide. It transmits a variety of pathogens, including spirochaetes that are the causative agent of Lyme borreliosis (LB) in humans. This study aimed at analysing different aspects of the ecology, molecular evolution, and microbial associations of *I. ricinus*.

A novel scheme for the phylogenetic analysis and genotyping of *I. ricinus* was established as part of this study. Phylogeographic clustering of *I. ricinus* samples from different European countries was observed, while samples from two different sites in Southwest England did not show spatial differentiation.

The ecology and host abundance in these two habitats was assessed. Differences in the density of questing ticks and in the abundance of rodent hosts were observed. Both sites exhibited low densities of rodents and of ticks infesting them.

A blood meal analysis revealed high proportions of mixed feedings and showed artiodactyls to be a main host group for immature *I. ricinus* ticks from Britain.

The prevalence rates of *Wolbachia* endobacteria in British *I. ricinus* samples were higher than rates found in samples from other European countries. The unique endosymbiont *Midichloria* was detected in all female *I. ricinus* ticks that were screened. A newly developed multi gene analysis of *Midichloria* samples from six European countries showed a largely coordinated phylogeography with their tick hosts.

Taken together, the findings of this thesis demonstrate that British *I. ricinus* ticks differ from their European counterparts in several ways, which has implications for the epidemiology of infectious diseases transmitted by this vector.

Table of contents

List of figures

Chapter 6:

List of Tables

Chapter 6:

List of abbreviations

12S	12S ribosomal RNA
µm	Micrometer
µl	Microlitre
AT	Allele type
ATP	Adenosine tri-phosphate
atp6	ATP synthase F0 subunit 6
BCE	Before common era
BLAST	Basic local alignment search tool
BMA	Blood meal analysis
BW	Bathampton Woods
cox1-3	Cytochrome C oxidase subunit I-III
cytb	Cytochrome B
dH_2O	Double filtered water
DLV	Double locus variant
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
GTR	General time reversible
HKY	Hasegawa, Kishino and Yano
IGS	Intergenic spacer region
l	Litre
LB	Lyme borreliosis
LGM	Last glacial maximum
LIV	Louping-ill virus
ml	Millilitre
mM	Millimol per litre
M	Mol per litre
$MgCl_2$	Magnesium chloride
ML	Maximum likelihood
MLST	Multilocus sequence typing
mtMLST	Mitochondrial multilocus sequence typing
n	Number of samples
nmol	Nanomol
NH_4OH	Aqueous ammonia

NJ	Neighbour-joining analysis
P	Probability
PCR	Polymerase chain reaction
RLB	Reverse line blot
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RW	Rainbow Woods
s.l.	*sensu lato*
s.s.	*sensu stricto*
SDS	Sodium dodecyl sulfate
SLV	Single locus variant
SNP	Single nucleotide polymorphism
ST	Sequence type
TAE	Tris acetate EDTA buffer
TBE	Tick-borne encephalitis
TROSPA	Ticks receptor of outer surface protein A
U	Unit of enzymatic activity
UV	Ultra violet light

1. General introduction

Ticks are obligate haematophagous (blood-feeding) ectoparasites[1] that feed on a variety of vertebrate host animals, including mammals, birds, reptiles, and sometimes amphibians. They transmit a wide range of disease-causing organisms and are of great medical and veterinary importance. This study will mainly focus on the biology of two species of *Ixodes* ticks, namely the sheep tick *I. ricinus* (L., 1758), and the blacklegged tick *I. scapularis*, Say 1821, as important vectors of Lyme borreliosis (LB) in humans.

1.1 A short history of ticks

Ticks have been known as a nuisance and a pest to mankind and animals for a long time. A depiction of ticks infesting a hyaena-like animal was found in an Egyptian tomb dating from about 1500 BCE (Arthur, 1965), when "tick fever" was also mentioned in a papyrus scroll (Varma 1993, cited in Hillyard 1996). Homer wrote about ticks (called "*kynoraistes*", or dogflies) in the *Odyssey* (ca. 850 BCE), while Aristotle in his *Historia animalium* associated tick infestation of animals with high grass, out of which it was believed ticks would originate. They were named "*ricini*", which would later give rise to the generic name of the castor oil plant, *Ricinus communis*, due to its seeds resembling ticks.

Pliny the Elder described ticks as "living on blood with its head always fixed and swelling", but believed that they had no anus and would therefore "burst with over-repletion and die from actual nourishment." (Arthur, 1965).

Later, a German encyclopaedia of natural history ("*Hortus sanitatis*" by Johann Wonnecke v. Kaub & Erhard Reuwich) from the 15th century would show the earliest depiction of a tick questing in the vegetation, together with sheep as one of its most common host (Hillyard, 1996). A more modern account was given by Kirby & Spence in their *Introduction to Entomology* (1815), in which they described in detail the act of blood feeding by a tick (cited in Medlock et al., 2009). In 1893, Smith and Kilbourne demonstrated for the first time that ticks transmit pathogens, in this case the protozoan

1: In the Attic democracy (ca. 500-300 BCE) priests would be elected to consume food that had been offered to the gods.The term "parasite" is derived from their name "*parasitos*" (greek for "one who eats at the table of another") (Enzensberger, 2001).

Babesia bigemina, causative agent of the Texas cattle fever, by the cattle tick, *Boophilus annulatus* (Assadian & Stanek, 2002).

1.2 General biology of Ixodes *ticks*

1.2.1 Tick classification

Ticks are arthropods closely related to mites, with which they form the subclass Acari within the class Arachnida. Arachnids differ from insects and other arthropods by their four pairs of legs (except in larval ticks), their two pairs of mouth-associated appendages (chelicerae and pedipalps), and their absence of antennae or wings (Hillyard, 1996). Members of the Acari are the only known parasitic arachnids. All ticks belong to the suborder Ixodida of the order Parasitiformes, as shown in Fig 1.1. Ticks can be distinguished from mites by their toothed hypostome, which is used by a tick to attach itself to the host while feeding, and by the sensory Haller's organ on the furthest part of the first pair of legs (Hillyard, 1996). See section 1.2.2 for a detailed description of tick morphology.

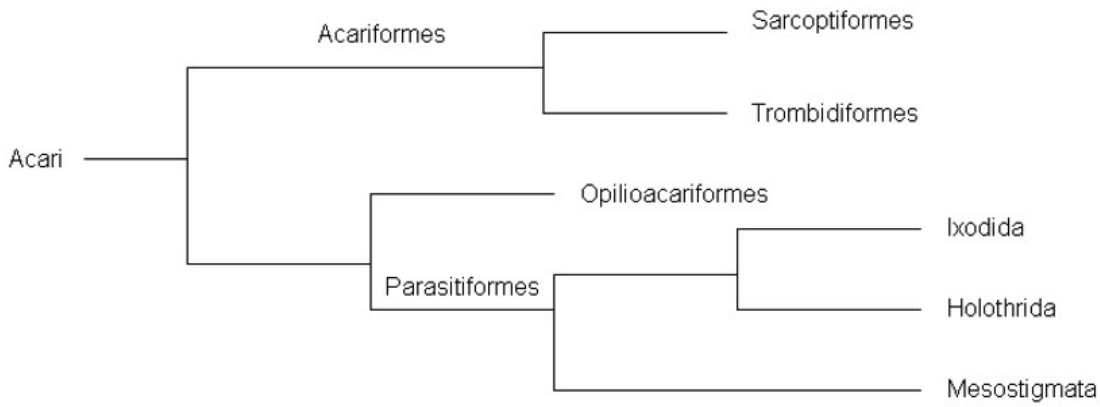


Fig. 1.1. General phylogeny of the Acari. Taken from Maddison & Schulz (2007).

The 896 known species of ticks can be further divided into three families, the Argasidae (soft ticks, 193 species), the Ixodidae (hard ticks, 702 species), and the Nutalliellidae (which consist of only one species from Africa, *Nuttalliella namaqua*) (Guglielmone et al., 2010). A current phylogeny of the Ixodida is depicted in Fig. 1.2. Hard ticks can be easily identified by their visible mouthparts, and by the presence of the scutum, a plate-like shield on the dorsal surface (Hillyard, 1996). They normally feed only once during each of the

three parasitic life stages (larva, nymph, adult female) and remain attached to the host for a long time (Gray, 2002). The name of the type species *Ixodes ricinus* is derived from the greek word "*ixos*" for the European mistletoe plant (*Viscum album*). Its sticky berries were commonly used since Roman times to make adhesive traps for birds, thus resembling the strong attachment of ticks to their hosts (W.A. Maier, pers. comm.).

Soft ticks lack a hardened upper surface and have less conspicuous mouthparts than ixodid ticks. They feed repeatedly, each time taking only small amounts of blood, and remain normally in the close vicinity of their hosts, such as in their nests and burrows (endophilic behaviour) (Gray, 2002). While some argasid species do transmit diseases (see Table 1.2 below), ixodid ticks are by far more important vectors for several zoonoses (infectious diseases that can be transmitted between animals and humans) and are considered the most important ectoparasites of livestock (Gray, 2002). Table 1.1 gives an overview of the main characteristics of hard ticks and soft ticks. Within the Ixodidae, a further distinction can be made between prostriate ticks (subfamily Ixodinae, consisting of the single genus *Ixodes* with 243 species) and metatstriate ticks (all other genera of hard ticks with 459 species), which is mainly based on morphological details (Guglielmone et al., 2010).

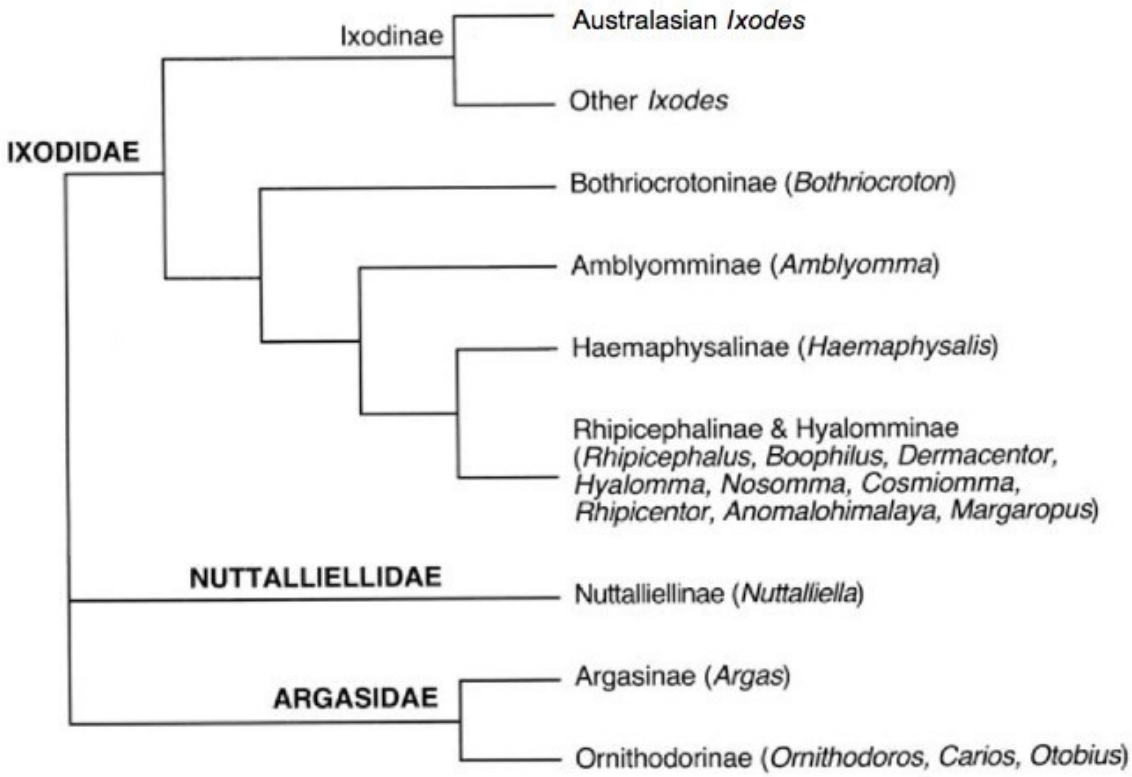


Fig. 1.2. Phylogeny of the families and subfamilies of the suborder Ixodida. Taken from Barker & Murrell (2004).

Following morphological comparisons, Filippova suggested to group 14 *Ixodes* species to one complex initially named *Ixodes persulcatus* group (Filippova 1969, 1971, 1973, cited in Filippova, 1991). After further molecular studies, this group has since been expanded to comprise currently 21 species which are globally distributed, and has also been referred to as *Ixodes ricinus* complex (Keirans et al., 1999; Xu et al., 2003), or as *Ixodes ricinus-persulcatus* group (Filippova, 2002). Fig. 1.3 depicts the molecular phylogeny of several members of the species complex within the Ixodinae. This complex contains all four *Ixodes* species (*I. ricinus*, *I. persulcatus*, *I. scapularis*, and *I. pacificus*) that are the main vectors for Lyme borreliosis (LB)-causing spirochaetes (see section 1.3).

Table 1.1. Comparison of hard ticks and soft ticks. Taken from Hillyard (1996).

	Hard ticks	**Soft ticks**
Sexual dimorphism	Well-marked in adults	Slight
Nymphal instars (stages between moults)	Single stage as nymph	From two to eight separate nymphal instars
Host relations	Many parasitise free-ranging hosts	Most dwell in nests and burrows of hosts
Number of hosts	Normally three hosts, often different species	Usually only one host species
Feeding	Each feeding stage feeds slowly and only once	Nymphs and adults feed several times and relatively briefly
Weight gain during feeding	Up to 120 times unfed weight	Less than 12 times unfed weight
Fertilisation	Mating usually a precondition of female engorgement	Engorgement does not depend on mating
Egg laying	Female lays thousands of eggs in single mass	Small clutches of eggs are laid over a period of time

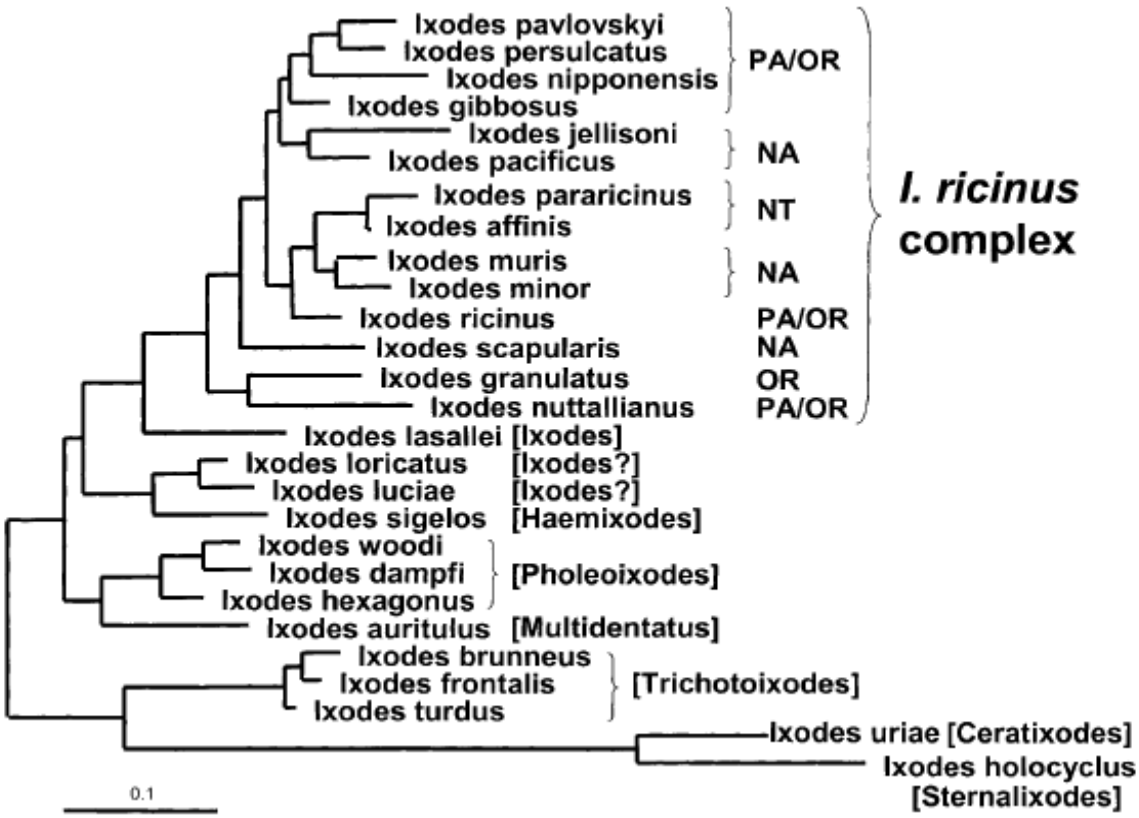


Fig. 1.3. Bayesian phylogenetic inference of 27 *Ixodes* species, based on 16S rDNA sequences. Eleven analysed species belong to the *I. persulcatus*/*I. ricinus* complex, with their geographic origins shown. Square brackets indicate subgenera, question marks indicate uncertain subgenera based on morphological characters. NA: Nearctic region; OR: Oriental region; PA: Palearctic region; NT: Neotropic region. Taken from Xu et al. (2003).

1.2.2 Tick morphology

This section is based on the detailed description of the external features of ticks by Hillyard (1996). The body of a tick consists of two parts, the capitulum and the idiosoma, which can be further divided into the podosoma (bearing the legs) and the opisthosoma (similar to the abdomen). The capitulum resembles a head and carries the mouthparts of a tick, but lacks any eyes. In ixodid ticks it is clearly visible, while it is much less conspicuous in argasids. Its basal portion is the basis capituli, and holds a pair of chelicerae, a pair of pedipalps (or palps), and one hypostome, which is toothed in order to maintain attachment to the skin of a host. On the dorsal surface of the basis capituli, female ixodid ticks possess a pair of porose areas, which contain the external openings of glands that secrete egg-protecting substances. The shape of the basis capitulum is used in the identification of tick species, based on structures that can project from the posterior corners of the dorsal side (cornua) or from the ventral side (auriculae) of the basis.

On their dorsal surface, ixodid ticks possess a shield, or scutum, which acts as a major distinction between hard ticks and soft ticks. In adult males it covers the entire dorsal surface, while in females and immature ticks it is restricted to the anterior part of the idiosoma, thereby allowing expansion during feeding of the remaining part of the idiosoma (known as alloscutum). If eyes are present in a tick species, they can be found on the sides of the scutum. Two pairs of grooves on its surface are used to aid in identification, while the scutum of some ticks, such as *Dermacentor* spp., can also be decorated by ornate markings.

The main features on the ventral surface are the first leg segments (coxa), respiratory spiracles that open into the tracheal system, and the genital and anal apertures. The genital aperture occurs only in adults and is surrounded by a genital groove, whereas the position of the anal groove can be used (in unfed ticks) to distinguish between prostriate (anal groove surrounds anal opening anteriorly) and metastriate ticks (groove is posterior to anus).

With the exception of the larval stages, all ticks possess four pairs of legs, each composed of six segments (coxa, trochanter, femur, patella, tibia, and tarsus). Both the presence and shape of internal and/or external spurs on coxa I and the profile of the tarsus I are used for identification. On the surface of tarsus I one can also find the Haller's organ, a unique complex of sensory pits and bristle-like sensilla that are crucial for the sensing of

external stimuli from hosts, such as changes in temperature, carbon dioxide concentration, humidity, ammonia or pheromones.

See Fig. 1.4 for a schematic depiction of an ixodid tick, and Table 2.3 for a detailed morphological description of six *Ixodes* species commonly encountered in Britain. *I. ricinus* can be distinguished from other tick species by the presence of porose areas on the dorsal capitulum, the absence of auriculae and of lateral spurs on the basis capituli, and by the elongated internal spurs on coxa I. It also has pedipalps that are relatively longer than wide, while its legs are of moderate length.

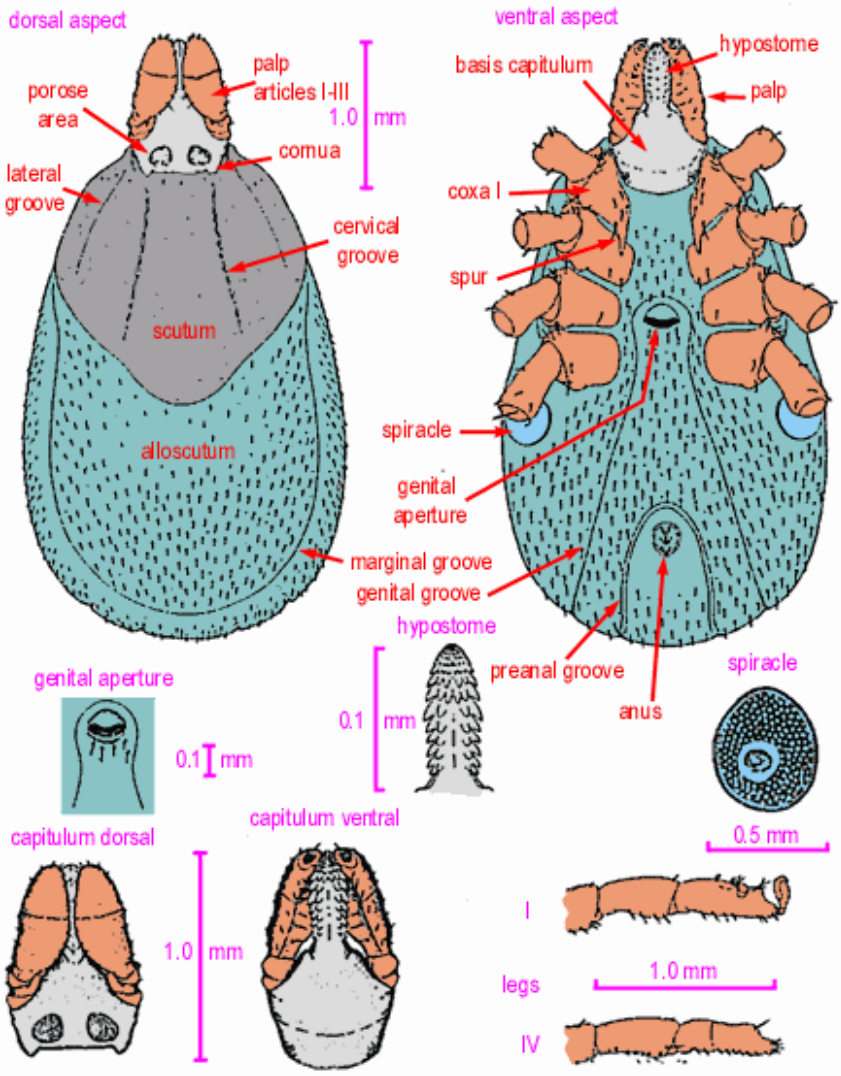


Fig. 1.4. General anatomic features of a female ixodid tick. Modified from Sonenshine (1997).

1.2.3 Life cycles of ticks

The life cycles of ixodid ticks, during which there are four stages (egg, larva, nymph,

and adult), take between one and six years. Each of the three parasitic stages seeks a hosts, attaches, feeds and drops off. Most ixodid species require a new hosts for each feeding stage (ie they are three-host ticks). Hosts may be confined to one single species or can come from a wide range of vertebrate animals, as in the case of *I. ricinus* (Hillyard, 1996).

Exophilic ticks such as *I. ricinus* actively seek their hosts by questing in the vegetation, whereas endophilic (nidiculous) species such as *I. hexagonus* remain in the nests or burrows of host animals to feed on them. Before the feeding act itself a tick may wander around while searching for a suitable position on the host's body. The duration of the feeding ranges from 2-6 days for larvae, 3-8 days for nymphs, and 6-12 days for adult females, while adult males rarely feed. In Metastriata and exophilic Prostriata, such as *I. ricinus*, mating occurs on their host, while endophilic prostriates mate off the host, mostly in the nest site (Hillyard, 1996). The feeding is followed by a digestion period and the moulting of larvae and nymphs to the next stage, while adult females begin to produce a single batch of 1,000-10,000 eggs, after which they die. In total, ixodid ticks spend only a small amount of time on their hosts, but over 90% of their life in long periods of resting and development, mostly hidden in the vegetation layer or in the host's nest. They are prone to desiccation and thus require protection against high temperatures and low levels of relative humidity (Hillyard, 1996).

Fig. 1.5 shows the life cycle of *I. ricinus*, including the duration of each moulting process, and groups of host animals that each stage most commonly feeds on. As indicated by the human symbol in the centre, each postembryonic stage is capable of infesting humans when encountered.

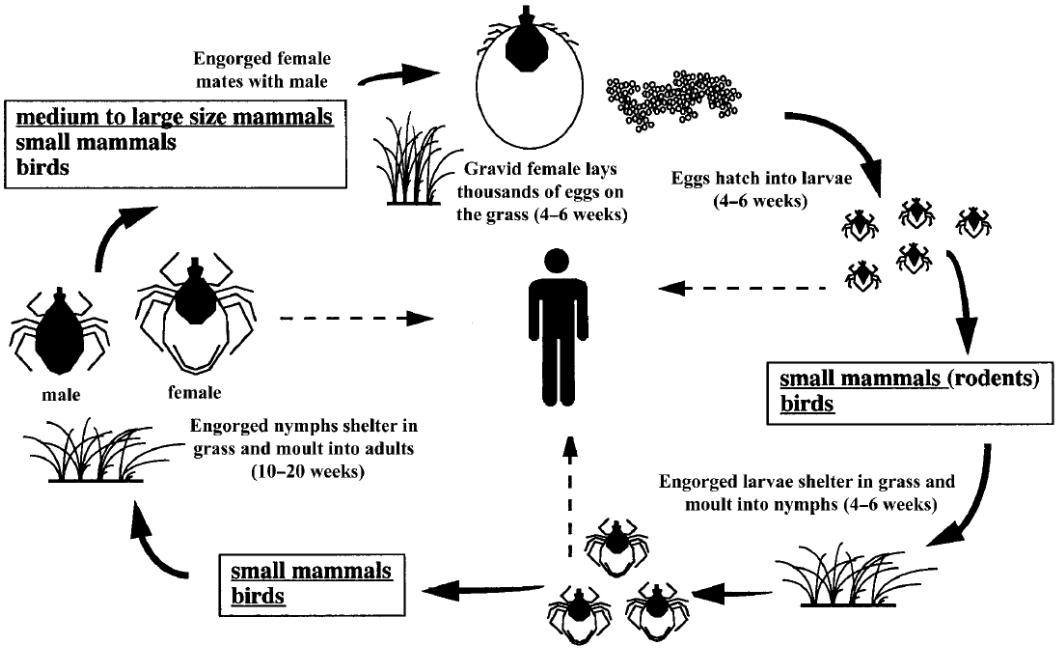


Fig. 1.5. Life cycle of *Ixodes ricinus*. Taken from Parola & Raoult (2001).

1.2.4 Geographical distribution and habitats of ticks

Ticks occur on every continent and are even found in marine colonies of seabirds across the Northern and Southern hemispheres (*I. uriae*, see Olsen et al., 1993).The four *Ixodes* species that are mainly responsible for the transmission of LB and other zoonoses are member of the *Ixodes ricinus-persulcatus* species complex and occur in temperate regions of the Northern hemisphere (see Fig. 1.6) (Gray, 2002). The Taiga tick, *I. persulcatus*, Schulze 1930, has the widest distribution of these four species, ranging from Poland in Central Europe across the entire region of boreal forests in Eastern Europe and Asia to Japan and Korea (Filippova, 1991; Keirans et al., 1999). The sheep tick, *I. ricinus* (L., 1758), occurs in temperate regions of Europe between the latitudes 65° N and 39° N, and extends eastwards from the British Isles (10° W) across Europe into Asia at a longitude of 60° E, partially overlapping with the distribution of *I. persulcatus* in Central and Eastern Europe (Gray, 1991). *I. ricinus* has also been found in North Africa (Yousfi-Monod & Aeschlimann, 1987, cited in Gray, 1991), although the taxonomic status of this population may require further evaluation (Noureddine et al., 2011).

In the Nearctic region, *I. pacificus*, Cooley & Kohls, 1943, ranges across the Western seaboard of North America from Southern Canada (British Columbia) to Northern Mexico,

and as far inland as Idaho and New Mexico (Keirans et al., 1999). The blacklegged tick, *I. scapularis,* Say 1821, can be found along the East coast of North America from Southern Canada (Nova Scotia, Quebec) southward to Florida, and westward to Texas (Keirans et al., 1999). It also occurs in the Midwestern US, but its distribution there is fragmented into three separate areas, each with a different LB epidemiology (see chapter 3.1.3).

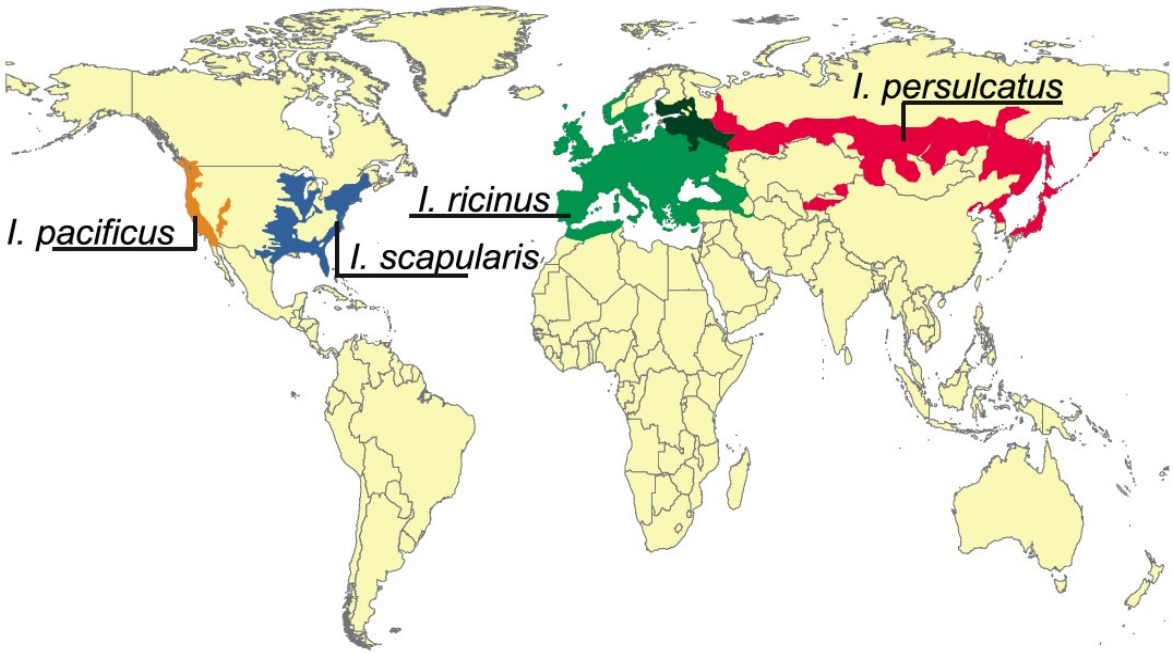


Fig. 1.6. Geographical distribution of four *Ixodes* species that are the main vectors for LB in humans. Taken from Swanson et al., 2006.

Habitats occupied by *I. ricinus* include deciduous and mixed woodlands, pastures, heathlands, and open grassland. Necessary requirements for its occurrence are suitable host animals, as well as high rainfall and dense vegetation to prevent desiccation (Gray, 2002; Hillyard, 1996). Due to higher levels of humidity on the British Isles, *I. ricinus* can also be found in meadows and exposed hillsides (Eisen & Lane, 2002; Medlock et al., 2008). Different tick stages tend to quest at different vegetation heights, most likely in response to desiccation stress (Randolph & Storey, 1999). A study in Sweden found that larvae of *I. ricinus* mainly aggregate at less than 30 cm above ground in both low and high vegetation types, whereas in high vegetation (herbal layer reaching 150 cm and more) nymphs and adults predominantly quest at 50-59 cm, and at 60-79 cm above ground, respectively. This stratification was less pronounced in low vegetation (0-50 cm), thus showing the adaptability of *I. ricinus* to biotic factors (Mejlon & Jaenson, 1997).

1.2.5 Hosts of *I. ricinus* and their role in tick distribution

Host specificity is measured by the degree of dependence of a tick species on a particular host species or group when these are available (Hoogstraal & Aeschlimann, 1982, cited in Hillyard, 1996). Host specificity in ticks can range from strict (as in the case of *I. lividus*, Koch 1844, which feeds exclusively on sand martins, *Riparia riparia*) to moderate (eg in the bat tick, *I. vespertilionis*, Koch 1844, which parasitises several species of bats) to the very broad and catholic host range of generalists, such as *I. ricinus* (Hillyard, 1996). For Northwest Europe alone, Hillyard (1996) listed over 55 different species and groups of host animals for all three stages of *I. ricinus*, while other hosts that are absent on the British Isles are also parasitised in mainland Europe, such as wild boar (*Sus scrofa*) or European elk (*Alces alces*) (Kjelland et al., 2011; Ruiz-Fons et al., 2006). The three stages of *I. ricinus* parasitise different host groups in different proportions, with larvae mainly feeding on rodents, such as wood mice (*Apodemus sylvaticus*) and bank voles (*Myodes glareolus*), nymphs on birds and medium-sized mammals, and adult females on large mammals, such as deer and livestock (Gray, 2002). It has been proposed that this differentiation in host infestations is the result of the stratified questing of the three stages in the vegetation, which would bring them into contact with different-sized host animals (e.g. Randolph & Storey, 1999). Regional variations in nymphal infestation rates on wood mice and in adult females biting humans have also been suggested to be due to behavioural differences between European *I. ricinus* populations (Gray, 2002).

Large mammals, such as deer and sheep, are considered vital to maintain tick populations in areas with favourable climatic conditions (Gray et al., 1992; Ogden et al., 1997). Together with migrating bird species they are also important for the distribution of ticks across long distances (Comstedt et al., 2006; Ogden et al., 2008; Ruiz-Fons & Gilbert, 2010). A recent increase in deer numbers (Fuller & Gill, 2001) and an expansion of their population ranges in the British Isles (Ward, 2005) has also been linked with an increase in the abundance and distribution of ticks (Scharlemann et al., 2008).

With regards to the transmission of LB spirochaetes (see next section), different hosts act as reservoirs for certain genospecies (a group of organisms that can interbreed) of the *Borrelia burgdorferi* s.l. species complex, while they inhibit the transmission of other genospecies. It has been shown that songbirds are associated with the genospecies *B. garinii* and *B. valaisiana* (Hanincova et al., 2003b), while rodents are associated with *B.*

afzelii (Hanincova et al., 2003a). Deer are considered resistant to LB spirochaetes (Jaenson & Talleklint, 1992; Telford III. et al., 1988), but rather act as maintenance hosts of tick populations, whereas sheep are capable of maintaining enzootic cycles of LB in the absence of other hosts (Gern et al., 1998; Ogden et al., 1997). Lizards (Lacertidae) are considered zooprophylactic for most LB genospecies, but act as reservoir hosts for *B. lusitaniae* (Vitorino et al., 2008).

1.3 Pathogens transmitted by Ixodes *ticks*

Apart from mosquitoes, ticks are the second most important vectors of human diseases worldwide, and the main disease vectors in the Northern hemisphere (Parola & Raoult, 2001). Globally they are also the most significant vector of diseases affecting livestock (Jongejan & Uilenberg, 2004). Ticks transmit a wider variety of pathogens than any other group of arthropod vectors, including viruses, bacteria (including spirochaetes, ie spiral-shaped bacteria with a flexible cell wall), and protozoans (Jongejan & Uilenberg, 2004). Hillyard (1996) listed several reasons why ticks are such efficient disease vectors:

- Firm attachment
- Slow feeding
- Wide host range
- Dispersal by hosts
- Adaptability
- Longevity
- High reproductive potential
- Resistance to starvation
- Salivary glands
- Suppression of host immunity
- Transovarial and transstadial transmission

Table 1.2 gives an overview of selected tick-borne diseases in humans, with those transmitted by *I. ricinus* shown in bold. This species, together with three other members of the *I. ricinus-persulcatus* group (*I. persulcatus*, *I. scapularis*, *I. pacificus*), is responsible for the transmission of several viral, bacterial, and protozoan diseases in humans. These include tick-borne encephalitis (TBE) in mainland Europe and Asia, human granulocytic anaplasmosis (HGA), Lyme borreliosis (LB), and babesiosis. TBE can be potentially fatal and causes over 10,000 cases of human infections each year in mainland Europe, but is notably absent on the British Isles (reviewed by Lindquist & Vapalahti, 2008). Here, the only endemic tick-borne viral disease is caused by the louping ill virus (LIV), which infects economically important game birds such as red grouse (*Lagopus lagopus scoticus*) in upland areas, and is maintained by mountain hares (*Lepus timidus*) and sheep in an enzootic cycle (Harrison et al., 2010). LB was first identified in and named after Old Lyme, Connecticut, USA (Steere et al. 1977, cited in Kurtenbach et al., 2006), and was later shown to be caused by a spirochaete transmitted by ticks (Burgdorfer et al., 1982) that was named *Borrelia burgdorferi*. It has become the most prevalent vector-borne disease both in Europe and in North America (Parola & Raoult, 2001), and case figures for both the United States and for the UK have been increasing over the last decades. Between 1995 and 2009, numbers of human LB cases in the US rose from 16,273 to 29,959 (CDC, 2011), while diagnosed cases in England and Wales reported to the Health Protection Agency (HPA) increased from 268 in 2001 to 973 in 2009 (HPA, 2011).

Molecular analyses of samples of borrelia strains causing LB showed that they form a species complex currently consisting of 17 identified genospecies (Margos et al., 2009; Rudenko et al., 2009, cited in Vollmer et al., 2011). These include *B. afzelii*, *B. garinii,* and *B. valaisiana*, which are the most abundant genospecies in Europe (Kurtenbach et al., 2001), and *B. burgdorferi* sensu stricto (s.s.), which predominates in the United States (Piesman & Gern, 2004). *B. afzelii, B. garinii*, and *B. burgdorferi* s.s. are human pathogens, but are associated with different manifestations of LB, such as skin manifestations (*B. afzelii*, see Canica et al. 1993, cited in Vollmer et al. 2011), neuroborreliosis (*B. garinii*, Rijpkema et al., 1997), and arthritic symptoms (*B. burgdorferi* s.s., Dam et al., 1993), while *B. valaisiana* is normally not associated with human disease (Wang et al., 1999).

Different genospecies have been found to be associated with different host groups (see

previous section), such as *B. afzelii* with small rodents and insectivores (Hanincova et al., 2003a), and *B. garinii* and *B. valaisiana* with songbirds (Hanincova et al., 2003b), whereas *B. burgdorferi* s.s. is considered a generalist species that infects both birds and rodents (Hanincova et al., 2006), and *B. lusitaniae* infecting lizards (Vitorino et al., 2008). As previously mentioned, deer are considered incompetent hosts that do not act as reservoir for LB spirochaetes, but rather as maintenance hosts for the tick populations (Jaenson & Talleklint, 1992; Telford III. et al., 1988). These host associations are the result of immune responses of the hosts to the spirochaetal infections, where the spirochaetes of non-compatible genospecies are killed by the complement arm of the innate immune response (Kurtenbach et al., 1998, 2002).

Kurtenbach et al. (2001) showed that the combinations of different borrelia genospecies vary strongly between different sites in Europe, while several studies revealed huge variations in Borrelia prevalence rates in ticks across Europe (e.g. Gassner et al., 2010; Kampen et al., 2004; Maetzel et al., 2005; Vollmer et al., 2011). These range from ca. 10% to 25% in mainland Europe, whereas nymphs on the British Isles exhibited lower prevalence rates of about 2-10% (summarised by Kurtenbach et al., 2006). It has been demonstrated that the structure of the host community in a habitat is shaping the abundance of the different genospecies of borrelia (Etti et al., 2003).

Borrelia are not transmitted from female ticks to their offspring (transovarially), but are rather taken up by ticks feeding on infected hosts. During moulting the spirochaetes remain in the tick gut and, once feeding commences, migrate to the salivary glands of the next stage (transstadial transmission), from where they can be passed on to another susceptible host. Therefore nymphs are the most important stage for the maintenance of an enzootic cycle of borrelia, since larvae are normally uninfected, whereas adult males do not feed, and adult females feed mainly on incompetent hosts such as deer (Gray, 2002; Kurtenbach et al., 2006). Co-feeding of ticks on infected hosts has also been observed (Gern & Rais, 1996; Ogden et al., 1997).

Table 1.2. Selected tick-borne diseases of humans. Diseases transmitted by *I. ricinus* are highlighted in bold. Modified from Piesman & Eisen (2008).

Disease	Causative agent	Main vector(s)	Geographic distribution
Viral			
Colorado tick fever	Coltivirus	*Dermacentor andersoni*	Western North America
Crimean-Congo hemorrhagic fever	Nairovirus	*Hyalomma marginatum*	Africa, Asia, Europe
Kyasanur forest disease	Flavivirus	*Haemaphysalis spinigera*	Indian subcontinent
Omsk hemorrhagic fever	Flavivirus	*Dermacentor marginatus, Dermacentor reticulatus, Ixodes persulcatus*	Asia
Tick-borne encephalitis	Flavivirus	*Ixodes persulcatus, Ixodes ricinus*	Asia, Europe
Bacterial			
African tick bite fever	*Rickettsia africae*	*Amblyomma hebraeum, Amblyomma variegatum*	Africa,West Indies
Human granulocytic anaplasmosis	*Anaplasma phagocytophilum*	*Ixodes ricinus, Ixodes pacificus, Ixodes scapularis*	Europe, North America
Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	*Amblyomma americanum*	North America
Lyme borreliosis	*Borrelia burgdorferi* s.l.	*Ixodes persulcatus, Ixodes ricinus, Ixodes scapularis, Ixodes pacificus*	Asia, Europe, North America
Mediterranean spotted fever	*Rickettsia conorii*	*Rhipicephalus sanguineus*	Africa, Asia, Europe
Q-fever[1]	*Coxiella burnetii*	Many species of different genera	Africa, Asia, Australia, Europe, North America
Rocky Mountain spotted fever	*Rickettsia rickettsii*	*Amblyomma cajennense, Dermacentor andersoni, Dermacentor variabilis, Rhipicephalus sanguineus*	North, South, and Central America
Tick-borne relapsing fever	*Borrelia* spp.	*Ornithodoros* spp.	Africa, Asia, Europe, North America
Tularemia	*Francisella tularensis*	Many species of different genera	Asia, Europe, North America
Parasitic			
Babesiosis	*Babesia divergens, Babesia microti*	*Ixodes ricinus, Ixodes scapularis*	Europe, North America

1): Transmission occurring via infected feces or coxal fluid rather than by the salivary transmission route.

1.4 Phylogenetics and mitochondrial multilocus sequence typing (mtMLST) of ticks

Phylogeny aims to trace evolutionary changes through successive generations of an organism. Phylogenetic studies can cover several time scales and different extents of evolutionary change, from small and potentially reversible changes within a species, to the formation of new species or even higher taxonomic groupings. Phylogeny is usually inferred by grouping together those organisms that share the greatest number of characters as related to each other (known as cladistics). The evolution of organisms involves changes in the DNA sequences of their genes (genotype), which may result in changes in their appearance and functional interaction with the biotic and abiotic environment (phenotype). In recent years, molecular approaches have become the preferred methods of phylogeny, usually utilising DNA sequencing to track changes in genotype directly, or other molecules, such as isoenzymes or cuticular lipids (Nei & Kumar, 2000; Page & Holmes, 1998). Although phylogeny traditionally examines ancestry in time, the same methodologies can be used to track the movements of groups of organisms in space (known as phylogeography, reviewed for example by Emerson & Hewitt, 2005).

Several studies have described the molecular evolution of different organisms in time and space, ranging from bacteria (eg reviewed by Maiden, 2006; Spratt, 1999), over trees (Tzedakis et al., 2002), sharks and rays (Douady et al., 2003), birds (Avise & Walker, 1998), marine mammals (May-Collado & Agnarsson, 2006), to arthropods (Jeyaprakash & Hoy, 2008; Regier & Shultz, 1997; Regier et al., 2010).

Mitochondria are small organelles of about 0.5 - 1.0 µm diameter that are involved in the generation of chemical energy in the form of ATP, metabolism, cell cycle and apoptosis of eukaryotic cells (reviewed by McBride et al., 2006). They are thought to have originated from free-living bacterial ancestors that were included into precursors of eukaryotic cells about 2 billion years ago, and were closely related to α-Proteobacteria (reviewed in Andersson et al., 2003; Gray et al., 1999). Through genome analysis it could be shown that *Rickettsia prowazekii*, the causative agent of epidemic typhus, is the closest living relative to mitochondria (Andersson et al., 1998). They are normally maternally inherited and their small genome is thought not to recombine (Birky, 2001). Mitochondrial genes have been shown to evolve faster than nuclear genes, for instance in the case of *Drosophila* four to nine times faster (Moryiama & Powell, 1997, cited in Ballard & Whitlock, 2004). These

characteristics, together with the fact that mitochondria occur in high numbers in each cell, make them suitable for phylogenetic analyses (Page & Holmes, 1998). Ticks possess a circular mitochondrial genome of variable size, for example 14,398 bp in the case of the soft tick *Ornithodoros moubata* (reviewed by Shao & Barker, 2007). Fig. 1.7 shows the arrangement of genes within the mitochondrial genome in non-Australasian *Ixodes* ticks: genes selected for a mtMLST scheme of *I. ricinus* are highlighted. Studies analysing the phylogeny of ticks have mainly employed sequences of the small and large rRNA subunits in mitochondria (also known as 12S rDNA and 16S rDNA, respectively) (Beati & Keirans, 2001; Black IV & Piesman, 1994; Mangold et al., 1998; Murrell et al., 1999; Norris et al., 1996). Further studies focusing on the phylogeography of tick populations have also used these loci (Caporale et al., 1995; Norris et al., 1996; Rich et al., 1995), as well as combinations of other mitochondrial genes (Casati et al., 2008; Noureddine et al., 2011). See also section 3.1 for a detailed description of these works.

Recent studies described the existence of an obligate intra-mitochondrial endosymbiont of ticks that is thought to be closely related to *Rickettsia* spp. (Beninati et al., 2004; Sacchi et al., 2004). Chapter 6 of this thesis analyses the prevalence and phylogeography of this organism in *I. ricinus* samples from different European countries.

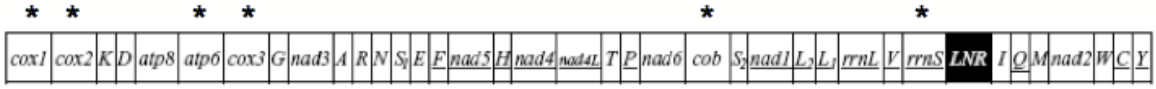


Fig. 1.7. Genome organisation of non-Australasian *Ixodes* ticks, including *I. hexagonus* and *I. persulcatus*. Protein-coding and rRNA genes are abbreviated as *atp6* and *atp8* (for ATP synthase subunits 6 and 8), *cox1–3* (for cytochrome c oxidase subunits 1–3), *cob* (for cytochrome b), *nad1–6* and 4L (for NADH dehydrogenase subunits 1–6 and 4L), and *rrnL* and *rrnS* (for large and small rRNA subunits). The transfer RNA (tRNA) genes are shown with the single-letter abbreviations of their corresponding amino acids. The 2 tRNA genes for leucine are *L1* and *L2* (uaa), and those for serine are *S1* (anti-codon sequence uag) (ucu) and *S2* (uga). *LNR* is the abbreviation for the large non-coding region. Loci used for the mtMLST scheme in *I. ricinus* are highlighted by asterisks. Modified from Shao & Barker (2007).

Bacterial populations have been studied through various methods (reviewed by Maiden, 2006) in order to study the distribution of emerging infections. Multilocus sequence typing (MLST) was initially developed by Maiden et al. (1998) to analyse the molecular epidemiology of the bacterial pathogen *Neisseria meningitidis*, and has since been applied to a wide range of bacteria and fungi (Enright & Spratt, 1999; Maiden et al., 1998; Meyer et al., 2009). It was based on multilocus enzyme electrophoresis (MLEE), a previously

developed typing method that assessed the electrophoresis of proteins derived from different alleles within a population. MLST uses nucleotide sequence data and is therefore more sensitive to mutations than a protein-based approach. Its main advantages are the reproducibility of standardised PCR protocols, easy exchange of data across the internet, and more robust analyses due to a larger sequence size (Maiden et al., 1998; Urwin & Maiden, 2003). According to guidelines defined by Maiden et al. (1998), an MLST scheme normally uses sequences of 6-7 (later expanded to up to 10 loci, see Maiden 2006) housekeeping gene (genes that are constitutively expressed in all cell types) fragments of approximately 400-600 bp length each. Criteria for the selection of suitable genes include: they should exist in single copies, evolve neutrally, are under stabilising selection, and do not undergo recombination. One important outcome of an MLST scheme is the creation of a super-sequence, obtained from the concatenated sequences of the amplified loci, which can be used for a more robust phylogenetic analysis than single-locus approaches. The second method used for the typing of samples is the assignment of type numbers to the different loci of an allele, eventually creating an individual allelic profile (or sequence type, ST) as a combination of these allele types (ATs). Analytic tools such as eBURST have been developed that enable samples to be allocated to clonal complexes, which may possess identical STs, or which differ by one, two or three loci, known as single locus variants (SLV), double locus variants (DLV), and triple locus variants (TLV), respectively (Feil et al., 2004).

Currently 27 MLST schemes for different organisms have been made accessible through the MLST website (www.mlst.net). These include an MLST analysis of *Borrelia burgdorferi* s.l. (Margos et al., 2008), which could identify a new borrelia species (*B. bavariensis*, Margos et al., 2009) and describe geographical and host-associated clustering (Vollmer et al., 2011).

Given the conflicting findings of various studies on the phylogenetic structure of *I. ricinus* populations in Europe, it was decided to develop a new mtMLST scheme for this parasite by employing sequences of several mitochondrial (mt) housekeeping genes (*atp6*, *cox1-3*, *cob*, see Fig. 1.7) and rDNA (12S) (see chapter 3 of this thesis). This was the main work carried out by my colleague Ruth Dinnis for her PhD project and is described in her thesis (Dinnis, 2010). A draft version of a manuscript describing the proof-of-principle of this method is currently under preparation and is attached as Appendix E of this thesis.

1.5 Overall aims and objectives of the sections in this thesis

In a broader context, an improved knowledge of the molecular evolution and phylogeography of *Ixodes* ticks may help to map the present risk of tick-borne diseases such as Lyme borreliosis. It could also aid in the prediction of future risks and how these will be affected by environmental changes such as increasing temperatures and precipitation patterns, and by shifting host distributions in different habitats (Diuk-Wasser et al., 2010). Future research might also include the development of genetically engineered vectors to control diseases transmitted by them (Sparagano & de Luna, 2008).

Taken together, the aim of this study was to provide an integrated approach to the multi-layered problematics of tick-borne diseases, with a special focus on Lyme borreliosis transmitted by *I. ricinus* in Europe. This included analysis of the molecular evolution of the ticks and of their endobacterial fauna, a description of their host and habitat ecology, and an attempt to quantify their host-seeking behaviour through a blood meal analysis. By combining molecular with ecological studies, it was aimed to gain further insight into the interactions between ticks and their environment.

The aims and objectives of each chapter of this thesis are listed below:

- **Chapter 1:** Give an overview of the biology of ticks, with a focus on the hard tick *Ixodes ricinus*, and pathogens transmitted by it.
- **Chapter 2:** Describe the materials and methods used in this work.
- **Chapter 3:** Describe the development of a novel mtMLST scheme to analyse phylogenetic processes in populations of *I. ricinus* in Europe. This work was undertaken in collaboration with Ruth Dinnis. Apply this scheme to samples of *I. scapularis* from North America.
- **Chapter 4:** Assess variations in host animal abundance, density and their tick infestation rates in two habitats with different ecologies near Bath, UK.
- **Chapter 5:** Analyse the composition of hosts parasitised by ticks by using a blood meal analysis (BMA) based on reverse line blotting (RLB). Compare the results of ticks from two British habitats with those from sites in Latvia, and differentiate between borrelia-infected and uninfected ticks.
- **Chapter 6:** Test the prevalence of several bacterial endosymbionts in ticks

collected in Britain. Compare the phylogeographic signals of a recently discovered mitochondrial endosymbiont, *Midichloria mitochondrii*, and its tick host *I. ricinus*, by using a multigene approach.

- **Chapter 7:** Provide an overall assessment and discussion of the findings obtained.

2. Materials and methods

The sections of this chapter describe in detail the methods used in this project. They also include “cooking recipe”-style lists of all chemicals and instruments used to allow other researchers to repeat these experiments. The sections are divided into methods that were carried out in the field, in the laboratory, and computer-based analysis methods.

2.1 Field methods

2.1.1 Assessment of habitat and vegetation types

Most parts of the field studies were carried out near the main campus of the University of Bath, UK. Two sites were studied throughout the period between 2006 and 2010 for questing ticks (see 2.1.2), and from 2009 to 2010 for small rodents (2.1.3). Figures 2.1 and 2.2 show a satellite image and a terrain contour map, respectively, of the area surrounding the campus of the University of Bath and denote the two study sites, Bathampton Wood (A), and Rainbow Wood (B). These two woodland sites were selected because of their vicinity to the campus (both could be reached on foot in 20 minutes) and public access. Previous study sites (not shown) had to be abandoned due to restricted access or disturbance by the general public.

Bathampton Woods (BW, 51° 23′ 1.43″ N, 2° 19′ 2.09″) is located on Bathampton Down, an oolitic limestone plateau that rises to a height of 204 metres above sea level and overlooks the valley of the river Avon (Ordnance Survey, 2008). The site consists of a wooded strip of ca. 200 m x 1000 m on a steep hillside slope, facing East-northeast. One major footpath crosses the area from North to South. The mines of the area have been declared a site of special scientific interest (SSSI) because of their populations of greater horseshoe (*Rhinolophus ferrumequinum*) and lesser horseshoe (*R. hipposideros)* bats (English Nature SSSI information sheet, 2011).

Rainbow Woods (RW, 51° 21’ 41.44″ N, 2° 20’ 38.17″ W) forms a roughly rectangular, 150 m x 400 m area, and is separated by stonewalls from Claverton Down Road in the South, private woodland in the East and West and borders open fields to the North. It forms a level area with few gullies and mounds of ca. 2-4 m height. It is intersected by small footpaths, which are frequented by the general public, especially dog walkers.

In both sites, dominant plant species and their visual cover were estimated for four different vertical layers: tree canopy, tree understorey/scrub, field and ground, and the

vegetation community was identified (Pigott *et al.*, 1991). Apart from the trapping of small rodents described below (2.1.3), other vertebrate host species, such as deer, sheep and songbirds, were identified, using the relevant field guides (Mullarney *et al.*, 1999, Harris & Yalden, 2008, Gurnell & Flowerdew, 2006).

The soil types of the two habitats were determined using the Soilscape website curated by the Geology Department of Cranfield University (http://www.landis.org.uk/soilscapes/).

Fig. 2.1. Satellite image of the area surrounding the main campus of Bath University (centre of image). A: Bathampton Woods; B: Rainbow Woods, shown by circles. Scale bar denotes 200 m. From Google Maps, 2011.

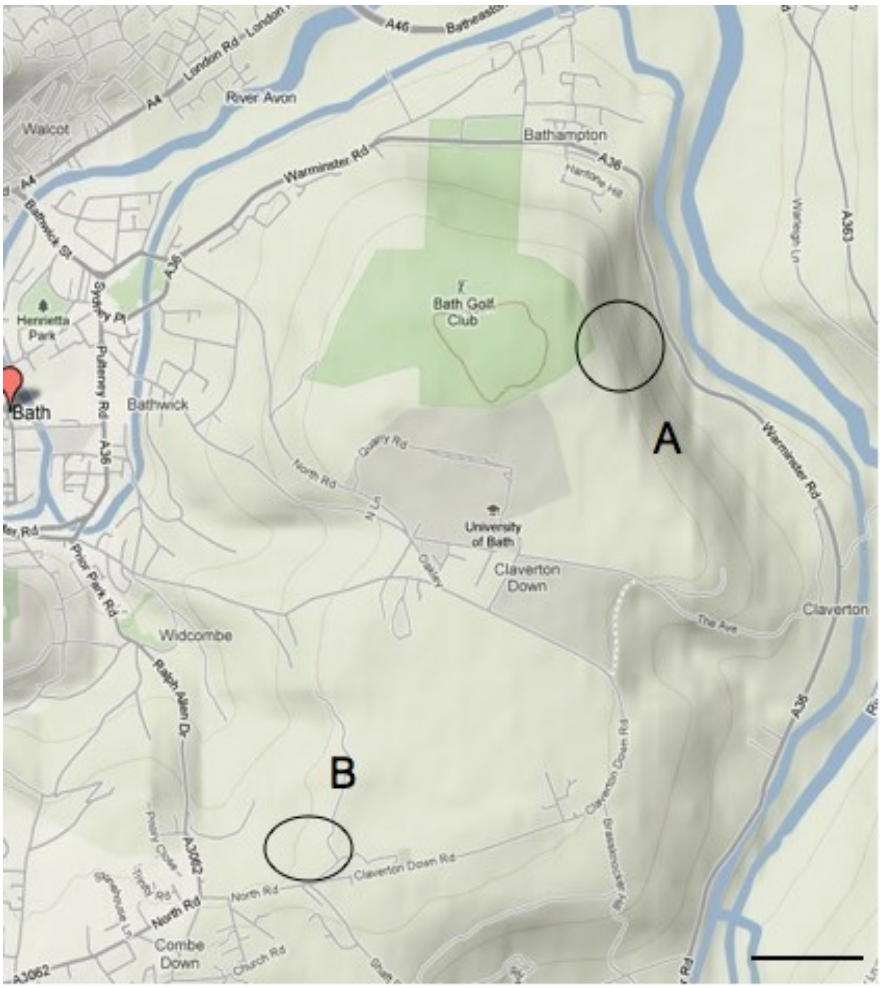


Fig. 2.2. Terrain contour map of the area surrounding the main campus of Bath University (centre of image). A: Bathampton Woods; B: Rainbow Woods, shown by circles. Scale bar denotes 200 m. From Google Maps, 2011.

2.1.2 Collection of questing ticks

- 70% ethanol (Fisher)
- 1.5 ml Safelock microcentrifuge tubes (Eppendorf)
- Light coloured cotton blanket, 1 m x 1 m
- Forceps
- RH85 handheld multisensor (Omega, UK)

The collection of questing ticks by cloth dragging was first used by Macleod (1932) and has been described by Hillyard (1996). Questing nymphal and adult ticks were collected by dragging a white cotton blanket of 1 m^2, which was attached to a wooden dowel, over the

vegetation. The blanket was checked for attached ticks after every 20 steps (ca. 10 m). Ticks were removed with forceps and placed in a 1.5 ml Eppendorf tube filled with 70% ethanol. The date, time, location and weather conditions were recorded each session, as were temperature and vegetation cover. Larval ticks were recorded but not collected.

The collections normally took place on dry days, either on late mornings or early afternoons. Ticks were collected near Bath, UK, between February and October from 2006 to 2010, and near Riga, Latvia, between March and September in the years 2002, 2006 and 2007. Tick collections in Latvia were conducted by Antra Bormane from the Latvian Health Protection Agency, Riga, and at the tick collection sites in England by Stephanie Vollmer, Ruth Mitchell and myself.

Further, less frequent tick collections took place near Leukerbad, Canton Valais, Switzerland in 2008, and around Bonn, Germany, and Lennestadt, Germany, in 2008 and 2009. We also received ticks that had been collected in Mafra and Grandola, Portugal, in 2003 from our collaborator Margarida Collares-Pereira of the Institute for Tropical Medicine and Hygiene, Lisbon, Portugal.

Adult *I. scapularis* ticks were collected from various regions in the United States by our collaborators and sent to our laboratory preserved in 70% ethanol. Ticks were collected in 2007 in Connecticut and Massachusetts in the Northeastern region of the US and in Wisconsin and Minnesota in the Midwestern US by Durland Fish (Yale University, New Haven) and his group. Andrea Varela-Stokes (Mississippi State University) provided ticks collected in 2006 in Mississippi in the South of the US.

Appendix A shows the geographical coordinates of these collection sites.

2.1.3 Trapping of rodents

- Bait (Rolled oats, peanut butter)
- Bedding material (hay)
- Fresh food (apples)
- 70% ethanol (Fisher)
- 1.5 ml Safelock microcentrifuge tubes (Eppendorf)
- Autoclave bag

- Callipers
- Forceps
- Ruler
- Scissors
- Electronic letter balance (Tanita, UK)
- Longworth live traps (Alana Ecology, UK)
- RH85 hand-held multisensor (Omega, UK)

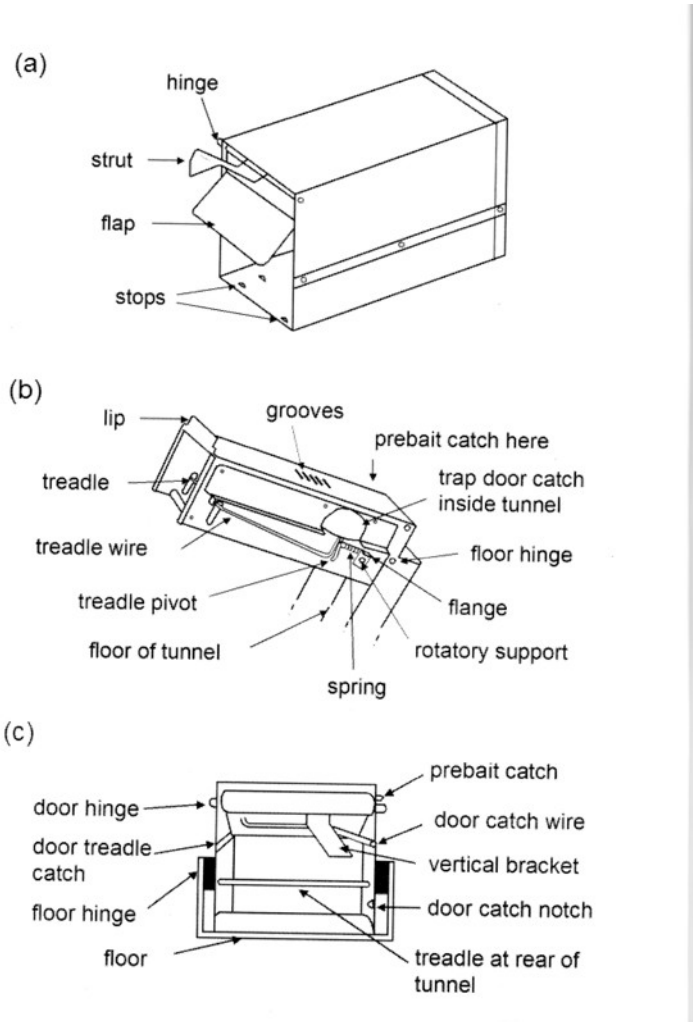


Fig. 2.3. Parts of a Longworth trap. (a) Nest box, (b) tunnel side view, (c) tunnel entrance, front view. From Gurnell & Flowerdew (2006).

The trapping of rodents was carried out following the instructions provided by Gurnell

& Flowerdew (2006). Longworth traps were designed by Chitty and Kempson in the 1940s and have become the standard trap type used for small mammals in Britain. The traps consist of two parts made from aluminium, an entrance tunnel with a door-tripping mechanism, and a nest box which is attached to the back of the tunnel in an assembled trap (Fig. 2.3). According to Section 11 of the Wildlife & Countryside Act (1981), it is prohibited to capture or kill shrews (Soricidae) without a licence. Therefore all traps used in our study were equipped with a small hole (ø 12 mm) to allow accidentally caught shrews to escape, and the treadle was set to a high tripping weight.

Between July and November 2009, and from April until October 2010, 25 traps were set up in each of the two sites described in 2.1.1. Trapping sessions were carried out monthly or fortnightly, depending on weather conditions, and lasted for two to four nights. To avoid high mortality of the captured animals, the trapping was halted during wet and cold weather periods. The traps were arranged in a rectangular grid, approximately 5 m apart from each other. Each trap was placed near natural obstacles, such as fallen logs or tree roots, and set at an angle so that the entrance was flush with the ground and stable to allow easy access by small mammals. The traps were baited with a mix of peanut butter and rolled oats, while pieces of apples were a source of moisture and dry hay provided bedding material for trapped animals.

After the trigger mechanism was set, more oats were spread near the trap entrances and the traps were covered with leaf litter to camouflage them.

Pre-baiting was carried out for one night before trapping sessions would commence. During the pre-baiting period, the trigger mechanism of the trap is arrested so that the trap door at the entrance of the tunnel remains open when the treadle is depressed by an animal. This allows the animals to get used to a new object in their habitat and to associate it with food despite its strong smell of humans.

Once the actual trapping begun, each trap was controlled every morning within 3-4 hours after dawn. Traps that had their doors shut were placed in a clear, strong polythene bag and carefully dismantled. The bag was used as a precaution against agile animals, such as wood mice, which can jump very high. Once the animal had left the trap, it was isolated in a corner of the bag, and the trap and any bedding material was removed. It was then gripped with the thumb and forefinger by the scruff of its neck, while the small finger was

used to secure the tail. The animal was lifted out of the bag and held closely to the chest to calm it down. Its species was determined by using a simple identification guide (Gurnell & Flowerdew, 2006) and it was examined for fur markings, breeding status and general body status. Some diagnostic features include the ratio of tail to body length, the fur colour and the body weight. Figure 2.4 shows the sexual characteristics of male and female rodents. The animal was carefully checked for the presence of ticks and of other ectoparasites by blowing gently against the fur and by examining the ears, snout and eye regions. If any ticks were discovered, they were pulled out with forceps and placed in a 1.5 ml safelock microcentrifuge tube filled with 70% ethanol. The total body length of the mammal and its hind foot length were measured and its body weight recorded. Before the animal was released, a small piece of fur was clipped off to mark the animal in a recapture, following a consistent pattern. The animals were marked from right to left, starting on the right hip, followed by the middle back and the shoulders, thus allowing for up to six clippings per animal. Due to legal reasons, no tissue samples, such as ear clippings, were obtained from the animals. These would have allowed testing for the presence of pathogens, such as borrelia.

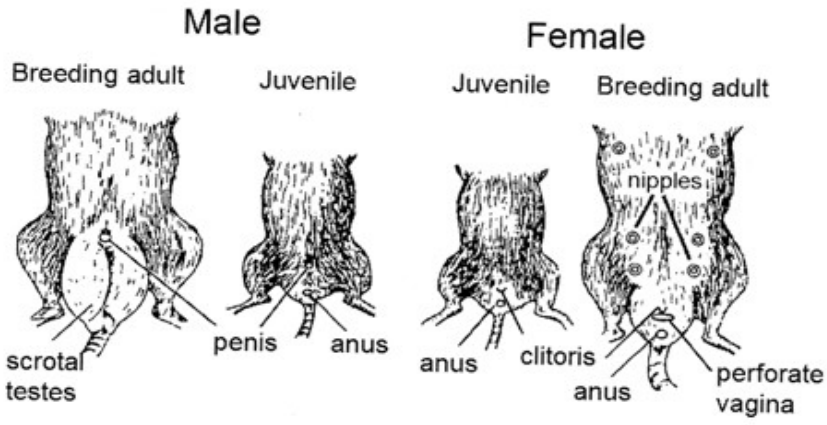


Fig. 2.4. Sexual characteristics of male (left) and female (right) rodents. From Gurnell & Flowerdew (2006).

Every morning during the trapping sessions, the general weather conditions were recorded and the temperature and relative humidity (RH) on ground level were measured using a hand-held multi-sensor. At the end of each session, the presence of questing ticks in the trapping area was examined by dragging a blanket (see section 2.1.2) 10 times for 10

m^2 through the vegetation, thus equalling 100 m^2.

2.1.4 Trapping of birds

To obtain information on the contribution of birds to the distribution of *Ixodes* ticks and Lyme borreliosis spirochetes, birds were caught and examined for ticks by experienced bird handlers (D. Wawman, M. Bailey) in two ringing stations. These stations were located on Chew Valley Lake (51° 20′ 4.92″ N, 2° 37′ 4.92″ W), approximately 21.5 kilometres Southwest of Bath, and in Bratton (51° 12′ 17.26″ N, 3° 30′ 38.94″ W) near Minehead, Somerset, on the Northeastern edge of Exmoor National Park, about 84 kilometres West-southwest of Bath. Birds caught with nets had their species and sex examined and were checked for ectoparasites. Special attention was given to the regions around the eyes, ears and the beak of the birds, as well as under the wings. Ticks were removed with forceps and stored in 70% ethanol for further analysis.

2.2 Lab methods

2.2.1 Identification of ticks

Both questing ticks and ticks collected from host animals were identified using morphological criteria using the classification keys provided by Hillyard (1996). Due to the large numbers of questing ticks that were collected, a subset was randomly chosen for specific identification. It can be assumed that due to their ecology the vast majority of all ticks collected by blanket dragging are *I. ricinus*, while other species with a nidicolous (nest-adapted) lifestyle, such as *I. hexagonus*, are very unlikely to be picked up (Milne, 1943). As ticks from caught wild animals can be parasitized by both nidiculous and non-nidiculous ticks, ticks that were collected from animals were all identified morphologically.

Ticks were classified according to stage, i.e. larva, nymph or adult. Adult ticks were further differentiated into males and females by the size of the dark scutum, which covers almost the entire dorsal surface in males (Fig. 2.5A), while it is confined to the anterior part of the idiosoma in females and immature stages, leaving the alloscutum clearly visible.

Other species of ixodid ticks were identified by their characteristic features described by Hillyard (1996) for the nymphal and adult stages and by Marquez *et al.* (1992) for the larvae. The species most likely to be found on wild vertebrate hosts from Britain include

the Southern rodent tick (*I. acuminatus*, Neumann, 1901), the passerine tick *I. frontalis* (Panzer, 1795), the hedgehog tick (*I. hexagonus*, Leach, 1815), the tree-hole tick (*I. arboricola*, Schulze and Schlottke, 1929) and the vole or shrew tick (*I. trianguliceps*, Birula, 1895). As indicated by their common names, most of these species exhibit a certain degree of preference for a vertebrate host or host group. Table 2.1 summarises characteristic morphological features of these species, and compares them with those of *I. ricinus*.

Table 2.1. Morphological features of six *Ixodes* species most likely to be found on non-domestic vertebrate hosts in Britain. After Hillyard (1996) and Marquez *et al.* (1992).

Tick species	Morphological characteristics of each stage
I. acuminatus (Southern rodent tick)	**Adult female:** Palps long and narrow, slender hypostome. Prominent cornua, posteriorly broadly rounded scutum. Auriculae prominent. Coxa I with prominent internal spur, external spurs on all coxae. Genital opening between coxae IV. Unfed 2.2-2.5 mm, engorged up to 6.0mm. **Adult male:** Unknown, no description available. **Nymph:** Long palps, prominent auriculae. External spurs on coxae I-IV, short internal spur on coxa I. Unfed 1.0-1.2 mm. **Larva:** Hypostome denticle formula 2/2. Cornua baso-ventrally angled.
I. arboricola (Tree-hole tick)	**Adult female:** Resembles *I. lividus* and *I. canisuga*. Short palps (articles II+III shorter than width of basis), short hypostome. Cornua reduced, scutum egg-shaped. Coxa I without internal or external spurs. Abrupt hump near apex on profile of tarsus I. Unfed 2.4-2.7 mm, engorged up to 6.0 mm. **Adult male:** Small capitulum, weak hypostome. Closely resembles female. Length 2.5 mm. **Nymph:** Short palps and hypostome. Scutum egg-shaped, all coxae without spurs. No auriculae, faint cornua. Short tarsus I with marked hump near apex. Unfed 1.2-1.4 mm. **Larva:** No auriculae, hypostome with subapical rows of 3/3 denticles. Seven pairs of margino-ventral setae.

Table 2.1. (continued)

Tick species	Morphological characteristics of each stage
I. frontalis (Passerine tick)	**Adult female:** Long palps, narrow and pointed hypostome. Scutum with characteristic hexagonal shape, longer than wide, posteriorly narrowly rounded. Blunt, button-like auriculae, likewise cornua. Coxa I with pointed internal and blunt external spurs. Long, tapering tarsus I, showing three dorsal humps in profile. Unfed 2.8-3.2 mm, engorged up to 8.0 mm. **Adult male:** Palps rather small, no division between articles II+III, moderate hypostome. Long, tapering tarsus I. Auriculae absent. No internal spurs on coxae I-IV. Length 2.1-2.3 mm. **Nymph:** Palps with slight outward curve, short hypostome; broad but short basis with acute-angled corners. External spurs on coxae I-IV well marked, internal spurs only on coxa I. Gradually tapering tarsus I. Unfed 1.5-1.7 mm. **Larva:** Articles I-III of pedipalps fused. Rectangular area of post-hypostomal setae. Lateral projections at basis.
I. hexagonus (Hedgehog tick)	**Adult female:** Palps shorter than width of basis, robust hypostome. Triangular cornua, but not prominent. Scutum characteristically hexagonal or heart-shaped. Genital opening between coxae III. Internal spur on coxa I narrow and pointed, as *I. ricinus*! Tarsus I clearly stepped near apex, unlike I. ricinus. Unfed 3.5-4.0 mm, engorged up to 1.3 cm. **Adult male:** Broad, oval body. Almost toothless hypostome. Prominent internal spur on coxa I. Tarsus I humped near apex. Length 3.5-3.8 mm. **Nymph:** Short palps, hexagonal scutum. Short or absent external spurs on coxae I-IV, internal spur on coxa I shorter than in female. No auriculae, but cornua apparent. Unfed 1.2-1.4 mm. **Larva:** Auriculae projecting. Eight pairs of dorsal and four pairs of ventral setae, dorsal setae longer than scutal setae.
I. ricinus (Sheep tick)	**Adult female:** Palps long and broad, cornua absent, auriculae reduced (Fig. 2.5C, D). Scutum slightly longer than wide, broadly rounded posteriorly. Coxa I, but not II-IV with long sharp internal spur (Fig. 2.5D). Tarsus I tapers gradually in profile (Fig. 2.5E). Genital aperture between coxae IV. Unfed 3.0-3.6 mm, engorged up to 1.1 cm. See Fig. 2.5B. **Adult male:** Palps broad and short, hypostome with prominent teeth. Pre-genital plate almost twice as long as broad, median plate much longer than wide. 2.4-2.8 mm. See Fig. 2.5A. **Nymph:** Palps long (articles II and III longer than wide). Scutum almost circular. Cornua and auriculae present, unlike adult female! External spurs present on coxa I-IV, internal spur on coxa I longer than external. Unfed 1.3-1.5 mm. **Larva:** Coxa I with rounded internal spur, slightly larger than external spur. Cornua perpendicular to basis, more or less prominent.

Table 2.1. (continued)

Tick species	Morphological characteristics of each stage
I. trianguliceps (Vole or Shrew tick)	**Adult female:** Clearly distinguished from other Ixodes species by lateral, triangular projection on base of palp (article I). Narrow palps, article II twice length of article III. Slender hypostome, teeth file-like. Only short protuberances as cornua. Scutum broad towards posterior, with small indents. Genital openings between coxae III. No internal or external spurs on coxa I. Short legs, tarsus I relatively long, no step between subapical hump and apex. Unfed 2.0-2.4 mm, engorged up to 8.0 mm. **Adult male:** Palps short, hypostome almost toothless. Coxa I lacks spurs. Legs short and slender. Length 1.8-2.2 mm. **Nymph:** Article I of palp with lateral/ventral spur. No coxal spurs, auriculae or cornua. Unfed 1.0-1.2 mm. **Larva:** Two pairs of post-hypostomal setae. Article I of pedipalps much larger than article II and with lateral expansions. Haller's organ closed.

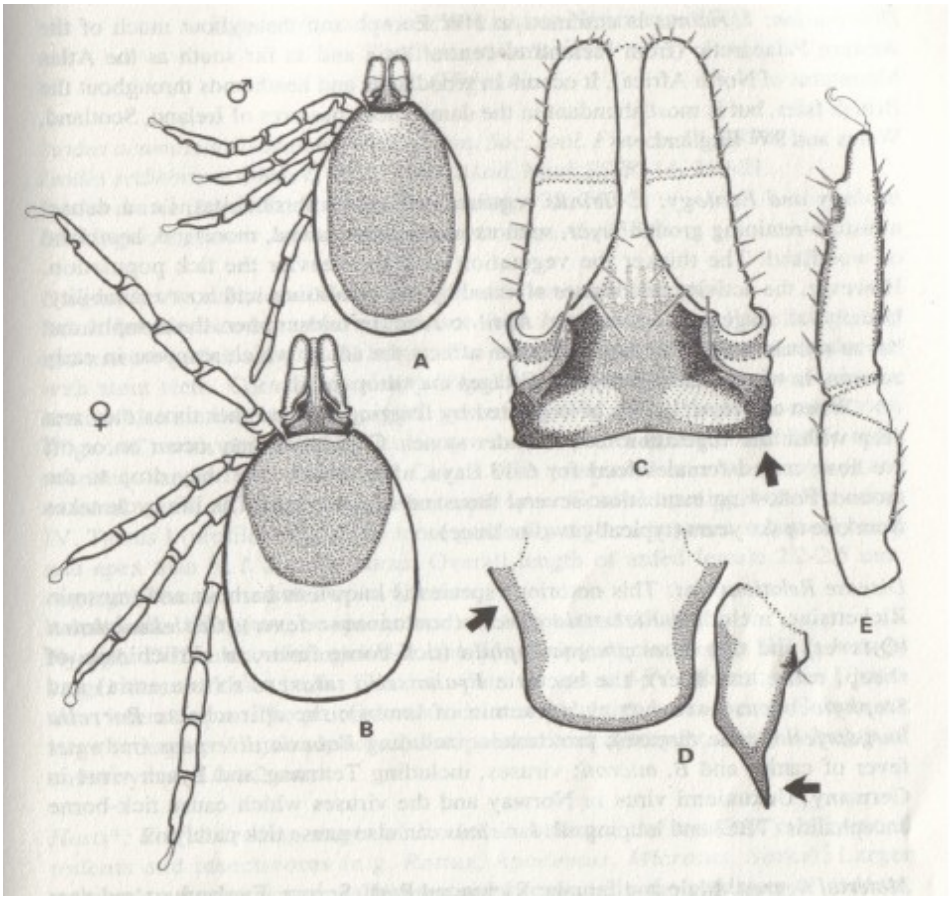


Fig. 2.5. Characteristic features of *I. ricinus* anatomy. A: adult male; B: adult female; C: Dorsal view of female capitulum, arrow indicates absence of cornua; D: Ventral view of female basis and coxa I, arrows indicate reduced auriculae (left arrow) and long, sharp internal spur (right arrow); E: Lateral view of female tarsus I. From Hillyard, 1996.

2.2.2 DNA extraction from tick samples

- NH_4OH (1.25%, Sigma)
- Heating block (Eppendorf, Hamburg, Germany)
- Microcentrifuge (Eppendorf)
- 1.5 ml Safelock microcentrifuge tubes (Eppendorf)

DNA from *I. ricinus* nymphs and adult ticks was extracted by alkaline hydrolysis (Guy & Stanek, 1991). The ticks were removed from their storage in ethanol or RNAlater with clean forceps and air-dried. One hundred microlitres of NH_4OH was added to nymphal ticks and 150 µl to adults in 1.5 ml Safelock microcentrifuge tubes. The cuticula was ruptured and the tissue was homogenized using disposable sterile pipette tips. The homogenized samples were heated to 100°C for 20 minutes on a heating block, removed, allowed to cool and placed back onto the block with the lids open to allow 50% reduction in volume through evaporation to remove remaining ammonia. Samples were stored at -20°C until further use.

2.2.3 Polymerase chain reaction (PCR)

- BioMix™ Red (BIOTAQ™ DNA polymerase, 2 mM dNTPs, 32 mM $(NH_4)_2SO_4$, 125 mM Tris-HCl, 0.02 % Tween 20, 4 mM $MgCl_2$, stabilizer, Inert dye) (Bioline, UK), concentration 2 x
- PCR primers (Invitrogen)
- $MgCl_2$ (QIAGEN)
- Template DNA

All primers were supplied in dehydrated and desalted form. They were rehydrated in sterile distilled water to a stock concentration of 100 nmol/l, which was further diluted to a working concentration of 10 nmol/l. PCR reactions were performed using BioMix™ Red at 1 x concentration. Two microlitres of template DNA and 10 pmol of each of the forward and reverse primers were added to each reaction. The total volume was made up to 25 µl with sterile dH_2O. Template DNA was extracted from ticks by the method described in section 2.2.2. The amount of template DNA was increased to 5 µl if a previous attempt had produced only a faint band on an agarose gel. Negative controls had tick DNA template

replaced with sterile distilled water. The specific primer concentrations and heating cycles for the different PCR protocols used in this study are shown in Appendix B.

2.2.4 Agarose gel electrophoresis

- Agarose (Invitrogen)
- TAE (Tris-acetate EDTA, Fisher)
- Electrophoresis power pack (BioRad, UK)
- loading buffer (Bioline, UK)
- DNA ladder (Bioline, UK)

Agarose gel electrophoresis was carried out using 1.5% agarose-TAE gels stained with ethidium bromide. Four microlitres of PCR product was loaded into each gel pocket. Gels were electrophoresed in 1 x TAE buffer solution at U = 100 V for 30 minutes and viewed using a UV-transilluminator.

2.2.5 DNA sequencing

Automated forward and reverse sequencing of PCR products was conducted by QIAGEN Genomics (Hilden, Germany) and by Agencourt, USA.

2.2.6 Blood meal analysis (BMA)

Following an initial scheme to analyze the blood meal of ticks described by Rijpkema et al. (1995), a more current protocol was developed by Humair et al. (2007), modified by Richard Birtles and Kevin Bown at the Department of Veterinary Pathology of the Faculty of Veterinary Science of the University of Liverpool, UK, and further adapted in our laboratory. It included the amplification by PCR of a ≈ 145 bp fragment of the mitochondrial 12S rDNA from vertebrate hosts on which the ticks had been feeding before moulting. A reverse line blot (RLB) was carried out to detect the binding of PCR products from individual ticks to specific vertebrate host probes (see section 2.3.2 for details of the probe designing).

Briefly, the RLB technique comprised the attachment of oligonucleotide probes specific to groups or species of vertebrate hosts to an activated membrane through a 5'-amino group attached to each probe. The 5'-amino group binds covalently to carboxyl groups on the membrane, while the oligonucleotide probes binds specifically to the biotinylated single-strand PCR products. The biotin label of the PCR products allows their detection on

a fluorescence-sensitive film through chemiluminescence. The oligonucleotide probes used in this study, as well as their nucleotide sequences and target organisms, are shown in Appendix C.

2.2.6.1 PCR

To identify the vertebrate hosts of previous blood meals in questing ticks, a fragment of approximately 145 bp length of the 12S rDNA from the host's mitochondria was amplified. The specific primer concentrations and PCR amplification steps used in this method are shown in Appendix B. Great care was taken to avoid contaminations. Extraction of DNA, PCR setup, addition of template DNA and post-PCR analysis (see 2.2.6.2) were carried out in separate areas. The areas dedicated to PCR setup, template DNA addition and post-PCR handling contained UV lamps and were regularly swiped with dH_2O to flush away any DNA. Each area also had its own dedicated set of pipettes and sterile filter tips.

2.2.6.2 Reverse line blot (RLB)

- 20 x SSPE stock (pH 7.4):

Substance	Amount (g/l)	Concentration (M)	Supplier
$Na_2HPO_4*2H_2O$	35.6	0.2	Sigma
NaCl	210.24	3.6	Fisher
EDTA	5.85	0.02	Sigma

- $NaHCO_3$ (0.5 M, pH 8.4, Fisher)
- 3 x SSPE/0.1% SDS, 500 ml, 50ºC (5 ml 10% SDS [BDH, Poole, UK], 75 ml 20 x SSPE, 420 ml dH_2O)
- 3 x SSPE/0.5% SDS, 55ºC (25 ml 10% SDS, 75 ml 20 x SSPE, 400 ml dH_2O)
- 3 x SSPE/0.5% SDS, 42ºC (25 ml 10% SDS, 75 ml 20 x SSPE, 400 ml dH_2O)
- 3 x SSPE, RT (75 ml 20 x SSPE, 425 ml dH_2O)
- EDTA (20 mM, pH 8)
- NaOH (0.1 M, Sigma)
- EDAC (16% w/v in dH_2O, Sigma)
- Streptavidin-Alkaline Phosphate conjugate (500 U/ml, Roche, Mannheim,

Germany)

- CDP-Star detection liquid (Roche)
- Biodyne C membrane (Pall Gelman)
- PC200 plastic support cushions (Web Scientific, Crewe, UK)
- Miniblotter 45 (Immunetics, Cambridge, MA)
- Amersham Hyperfilm ECL (GE Healthcare)
- Film cassette 18 x 24 cm (X-OGraph, UK)
- Automated film developer
- Cling film

The oligonucleotide probes were supplied in dehydrated and desalted form and were dissolved in 150 µl $NaHCO_3$ to a stock concentration of 100-500 nmol/l. They were further diluted to a working concentration of 100-500 pmol/l by adding 1 µl of stock solution to 150 µl $NaHCO_3$.

A Biodyne C membrane was activated by incubation in 15 ml freshly prepared EDAC solution for 15 min in a plastic container at RT. It was washed with dH2O for 2 min and placed on a support cushion in a miniblotter system. The first lane of the miniblotter was filled with 150 µl drawing ink, followed by 150 µl of oligonucleotide solutions per lane. The membrane was incubated for 10 min at RT and the oligonucleotide solutions were removed by aspiration. The membrane was removed from the miniblotter with clean forceps and inactivated for 10 min in NaOH on a shaker at RT. It was rinsed twice with dH2O and washed by gentle shaking in 250 ml 3 x SSPE/0.1% SDS for 10 min at 50ºC. The membrane was subsequently washed in 100 ml EDTA for 15 min at RT, wrapped in cling film and stored at 4ºC.

One hundred and thirty microlitres of 3 x SSPE/0.1% SDS was added to each well of biotinylated PCR products. The diluted PCR products were heat-denatured for 10 min at 100ºC and immediately cooled on ice. The membrane was washed for 5 min in 250 ml 3 x SSPE/0.1% SDS at 50ºC and then placed on a support cushion in the miniblotter. The membrane was placed perpendicular to the applied oligonucleotide solutions, with the ink line below the top row of holes for the blotter lanes. Each slot was filled with 150 µl of

diluted PCR product and hybridised for 60 min at 50ºC without shaking. The samples were removed by aspiration and the membrane was washed twice with 250 ml 3 x SSPE/0.5% SDS at 55ºC for 10 min. Five microlitres of Streptavidin-AP conjugate was added to 25 ml of 3 x SSPE/0.5% SDS and the membrane was incubated in this solution for 30 min at 42ºC while shaking. To remove excess Streptavidin-AP, the membrane was washed twice in 250 ml 3 x SSPE/0.5% SDS at 42ºC for 10 min, followed by rinsing it twice with 3 x SSPE for 5 min at RT to remove SDS.

To detect hybridized DNA, the membrane was incubated for 5 min at RT in CDP-Star detection liquid with slight shaking. Afterwards excess liquid was poured off and the membrane was wrapped in cling film. Great care was taken to avoid air bubbles to ensure good contact with film. The membrane was placed in a film cassette and exposed to a sheet of Amersham Hyperfilm ECL in a dark room for 15 min, followed by automated developing.

2.3 Computer-based methods

2.3.1 Primer design

Primers for the following genes were previously designed by Ruth Mitchell: ATP synthetase subunit 6 (*atp6*), cytochrome oxidase subunit I (*cox1*), cytochrome oxidase subunit II (*cox2*), and cytochrome oxidase subunit III (*cox3*). Primers for cytochrome b (*cob*) and 12S subunit rRNA (*rrnS*) were designed by myself. The published sequences of the respective mitochondrial genes from the Eurasian taiga tick *I. persulcatus* (http://www.ncbi.nlm.nih.gov/sites/entrez, genome accession number NC_004370) were inserted into the freeware interface Primer3 (http://primer3.sourceforge.net/), using its default parameters for primer pairs at approximately 500 base pairs apart. Primers were then synthesized by Fisher Scientific UK and were supplied in desalted and dehydrated form.

2.3.2 Mitochondrial multilocus sequence typing scheme (mtMLST)

As described in chapter 1.4, a mitochondrial multilocus sequence typing (mtMLST) scheme was developed in our group. A detailed description of the methodology employed for this mtMLST scheme can be found in the manuscript provided in Appendix E. Briefly, after DNA had been extracted from collected ticks (see section 2.2.2), fragments of about

450-550 bp length of five mitochondrial genes (*atp6*, *cox1-3*, *cob*) and 12S rDNA (*rrnS*) were amplified by PCR (see 2.2.3). Following sequencing and error checking, these sequences were aligned (see 2.3.3 below) and used for further analyses. These included the assignment of sequence types (STs), and subsequently, allele types, by entering the sequences into a non-redundant data base (NRDB) available online (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=nrdb&referer=pubmlst.org). The aligned sequences were also concatenated to form super-sequences comprising all six loci that were then used for phylogenetic analysis (see 2.3.4).

2.3.3 Alignment and editing of sequenced DNA trace files

The received forward (5'→3') and reverse (3'→5') DNA traces were trimmed to conserved motifs using the Lasergene SeqMan software (DNAstar, Madison, Wisconsin, USA). The obtained consensus sequences were checked for errors by using the automated ambiguous base matching function of the program and by manually checking bases of poor quality. The consensus sequences were aligned with MEGA version 4 (Tamura *et al.*, 2007), using the default parameters for ClustalW alignments.

2.3.4 Phylogenetic analyses

The built-in function of MEGA5 was used to select suitable models (GTR and HKY) of molecular evolution (Tamura et al., 2011). Neighbour-joining (NJ) (Saitou & Nei, 1987) and Maximum-Likelihood (ML) (Hasegawa et al., 1985) trees were created using MEGA4 and MEGA5 (Tamura et al., 2007; 2011). Phylogenetic inferences were processed using MEGA5 and Dendroscope (Huson et al., 2007).

2.3.5 Oligonucleotide probe design

Of the 40 oligonucleotide probes described by Humair et al. (2007) for their blood meal analysis, 38 were used in this study. The probe for *Parus ater* (coal tit) was excluded to allow further probes of more significant host species to be used. The probe for *Homo sapiens* (human) had to be omitted since contamination with human DNA could not be avoided in all cases. Instead, six more probes specific for host species were designed by our group. These species included: *Ovis aries* (common sheep), *Cervus elaphus* (red deer), *Dama dama* (fallow deer), *Oryctolagus cuniculus* (European rabbit), *Passer domesticus* (house sparrow) and *Phasianus colchicus* (common pheasant). The published sequences of the 12S rDNA of these species were retrieved from GenBank

(http://www.ncbi.nlm.nih.gov/sites/entrez) and were aligned in MEGA4 (Tamura *et al.*, 2007). Regions that showed variability and were unique to single species were used to design new oligonucleotide probes using Primer3.

2.3.6 Statistical analysis

Statistical tests were carried out using the R software package (version 2.9.2 for Mac OS X, 2009), which can be freely downloaded from the internet (http://www.R-project.org).

3. Phylogenetic analysis of *Ixodes* tick populations in Europe and North America

3.1 Introduction

3.1.1 Importance of phylogenetic studies on *Ixodes* ticks as Lyme borreliosis vectors

Previous studies have analysed the phylogenetic relationships of arthropod vectors of human and animal diseases, such as mosquitoes (Cywinska et al., 2006), ticks (Burlini et al., 2010), and tsetse flies (Dyer et al., 2011) in order to assist in their surveillance and control. These studies may help to understand differences in pathogen transmission and in host-association between different populations, which in turn have serious implications for the correct prediction of disease-associated risks. The population structures of *Ixodes* ticks as the main vectors for Lyme borreliosis (LB) have also been studied using different molecular approaches. In Europe, Estrada-Pena et al. (1998) described 10 different "strains" of *I. ricinus* based on an analysis of cuticular hydrocarbons, while Delaye et al. (1997) used allozyme data to compare Swiss samples of *I. ricinus* from two geographically separated sites and found no evidence of genetic isolation. Similar findings were made by Casati et al. (2008), who compared tick samples from several European countries by using mitochondrial sequences, but again did not observe any strong phylogeographic differentiation between these populations (for a detailed discussion of this study, as well as those by McLain et al., 2001; de Meeus et al., 2002; and Noureddine et al., 2011, see section 3.3.2 below). *I. ricinus* populations on the British Isles have been shown to display differences in their host associations (Gray et al., 2000) and in their transmission potential for LB spirochaetes (Vollmer et al., 2011) compared to tick populations from mainland Europe, which highlights the need for a biogeographic comparison of these tick populations.

In North America, fragments of the 16S mitochondrial gene have been employed by Caporale et al. (1995), Rich et al. (1995), Qiu et al. (2002) and Humphrey et al. (2010) to analyse populations of *I. scapularis*, which revealed two distinct clades (see section 3.1.3 for a detailed overview).

3.1.2 Multilocus sequence typing (MLST) as a novel tool to determine the phylogeographic structure of populations

Multilocus sequence typing (MLST) was originally developed by Maiden et al. (1998) to type and identify virulent strains of the bacterium *Neisseria meningitidis* and has since

been applied to several other species of bacteria and pathogenic fungi (reviewed by Maiden, 2006; Urwin & Maiden, 2003), including LB spirochaetes (Margos et al., 2008, 2009; Vitorino et al., 2008). Its major advantages are reproducibility, the ability to exchange data via the internet, and delivering more robust findings than studies that rely on single gene markers. The main criteria for choosing potential loci for a novel mtMLST scheme are that the genes should exist in single copies, evolve (nearly) neutrally, and do not undergo recombination. Mitochondrial genes in Eukaryotes do fulfill all of these criteria, which led our group to develop a novel mtMLST scheme for *I. ricinus* populations that consisted of five fragments of mitochondrial housekeeping genes (*atp6*, *cox1-3*, *cytb*) and 12S rDNA (see Dinnis, 2010, and Appendix E).

3.1.3 *I. scapularis* populations in North America have differential transmission potentials for LB spirochaetes

According to data from the Centers for Disease Control and Prevention (CDC), the number of confirmed cases of human LB in the United States has increased between 1995 and 2009 from 16,273 to 29,959 cases per year, making LB the most common vector-borne disease in the US, occurring in 12 different states (CDC, 2011). Therefore it is not surprising that the phylogenetic and phylogeographic status of *I. scapularis*, Say 1821, the main vector of human LB in the Eastern parts of the US, has received significant attention. *I. scapularis* has been shown to expand its distribution range, as can be seen in a comparison of established and new foci in the 20th century displayed in Fig. 3.1 (Dennis et al, 1998). For several years the Northern population of *I. scapularis* was identified as a separate species (*I. dammini*, Spielman et al. 1979), considered to be distinct from the more southerly distributed *I. scapularis* (Spielman et al., 1979). However, Oliver et al. (1993b) could demonstrate their conspecificity in reciprocal cross-breeding experiments, in which these two supposedly different species could produce fertile offspring through the F_3 generation, which confirmed findings of a molecular study using ribosomal DNA (Wesson et al., 1993). Since then, many studies have attempted to clarify the population structure of this species using different molecular markers. Caporale et al. (1995), Rich et al. (1995), Qiu et al. (2002) and Humphrey et al. (2010) employed 16S mt rDNA, while Norris et al. (1996) used a combination of 12S and 16S sequences. These studies could confirm the existence of two distinct clades, one Northern clade (sometimes referred to as “American” clade, (Norris et al., 1996) occurring in the Northeastern and Midwestern States, and a

Southern clade with a distribution ranging from Virginia to Florida, and from Texas to Arkansas (see Fig. 3.1). Immature ticks from the Southern clade have been shown to feed mainly on lizards (eg Apperson et al., 1993; Durden et al., 2002; Spielman et al., 1984), whereas immature stages of the Northern clade tend to feed on small mammals such as white-footed mice (*Peromyscus leucopus*) (Piesman & Spielman 1979, cited in Norris et al., 1996). These differences in host-association have implications for the epidemiology of LB in the Southern states of the US. Although ticks from the Southern clade have been shown to be competent vectors for LB spirochaetes *in situ*, they exhibit much lower prevalence rates for these pathogens in the wild than ticks from hyper-endemic areas in the North, possibly due to refractory effects of lizards to borrelia infections (reviewed by Oliver, 1996). They also rarely transmit these pathogens in the wild to hosts, including humans (Luckhart et al., 1992), which results in far fewer cases of human LB in the Southern States of the US (see Fig. 3.2). It has been demonstrated by Qiu et al. (2002) that ticks from the Northeastern clade exhibit genetic signs of recent colonization and rapid expansion, and that their origin could be traced back to few individuals originating in the Southern clade. This would suggest the recolonization of Northern areas by ticks from Southern refugia after the end of extensive glaciation in the pleistocene (Hewitt, 2004; Qiu et al., 2002), most likely via their mammalian and avian hosts. These hosts may also have helped in the more recent introduction of *I. scapularis* populations to the Midwestern region, as hypothesized by Jackson & DeFoliart (1970, cited in Humphrey et al., 2010), while other studies suggested continuous long-distance migration of birds as the main source of tick introductions and increasing tick densities (Ogden et al., 2008). Gatewood et al. (2009) revealed that the host-seeking activity of immature *I. scapularis* in the Midwestern and Northeastern populations varied as a result of climatic differences. These findings show a substantially different evolutionary origin of tick vectors in North America compared to the origin of LB spirochaetes, which may have originated in Europe and were probably introduced into North America in the Northeastern region (Hoen et al., 2009; Margos et al., 2008; Qiu et al., 2008). Taken together, these observations highlight the need for an integrated study on the ecology and phylogeography of *I. scapularis* populations.

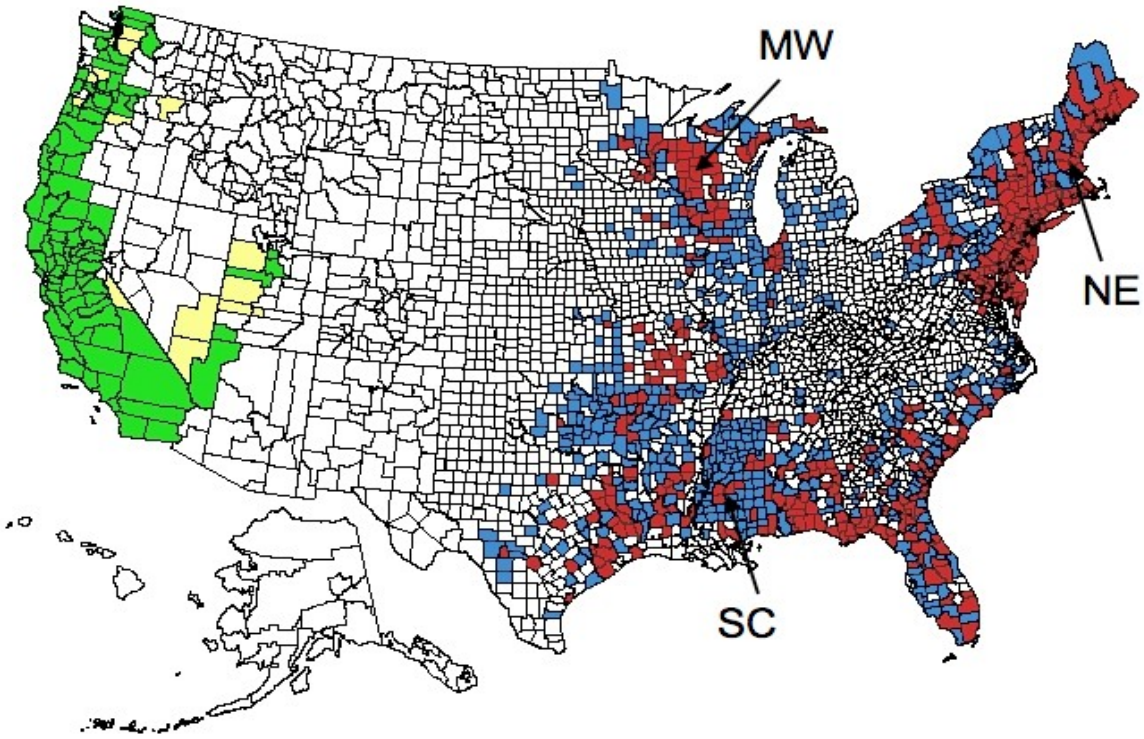


Fig. 3.1. Map of established (at least six individual ticks or ticks from two different stages to be identified in this county) and reported (at least one tick was identified in the county) distribution of the Lyme disease vectors *Ixodes scapularis* and *I. pacificus*, by county, United States, 1907-1996. Red = Established *I. scapularis*; blue = Reported *I. scapularis*; green = Established *I. pacificus*; yellow = Reported *I. pacificus.* Arrows indicate approximate collection locations of *I. scapularis* samples included in this study. MW = Midwestern clade; NE = Northeastern clade; SC = Southern clade. Modified from Dennis et al., 1998.

Fig. 3.2. Geographical distribution of reported human cases of Lyme disease in the continental United States in 2009. One blue dot was placed randomly in county of residence for each reported case. From CDC, 2009.

The aims of the work undertaken in support of this chapter were as follows:

- Establish a novel, mtMLST scheme for the phylogeographic analysis of *I. ricinus* populations from Britain and mainland Europe (this work was carried out jointly with Ruth Dinnis).
- Test whether *I. ricinus* ticks collected from different locations near Bath, UK, exhibit phylogenetic differences.
- Analyse *I. ricinus* ticks from two different countries (UK and Latvia) for phylogenetic differences.
- Apply this mtMLST scheme to *I. scapularis* ticks from three different North American clades.

3.2 Results

3.2.1 My contributions to the results of this chapter

I was involved in establishing a novel mtMLST scheme for populations of *I. ricinus* from the British Isles and from mainland Europe. My main contributions to this project consisted of extracting DNA from tick samples from Britain, Latvia, Portugal and Germany, and their subsequent testing by PCR for the six gene fragments included in the scheme. I also developed two sets of novel primers to amplify fragments of the 12S and the *cyt b* genes that were included in the final mtMLST scheme and tested primers for potential loci, such as *nad4* and *nad5*. Furthermore, I edited the raw sequences and manually checked the sequence trace files for possible errors. In order to confirm that the sequence editing techniques developed in our group were working properly, I double-blind tested samples from Britain and Latvia by repeating the PCR processing, sequence editing and assignment of allele types without prior knowledge of their original status. The results could be entirely replicated, thereby confirming that our approach was functioning. All results obtained from ticks analysed under this scheme were included in a database curated by Dr. Dinnis and myself.

A full description of the results generated from this novel scheme can be found in the PhD thesis of Dr. Dinnis (Dinnis, University of Bath, UK, 2010) and in a paper currently being processed (Dinnis et al., in preparation, see Appendix E for a current version of the

manuscript).

3.2.2 Comparison of ticks from two habitats near Bath, UK

3.2.2.1 Comparison of ticks collected in 2007 and 2008 from BW and RW

In order to test whether ticks collected in Bathampton Woods (BW) and in Rainbow Woods (RW) showed any phylogeographical structuring, the concatenated mtMLST sequences of 40 randomly selected ticks from BW and RW were analysed. These consisted of 10 ticks each from 2007 and 2008 from both BW and RW. A phylogenetic reconstruction using the Neighbour-Joining (NJ) method (Saitou & Nei, 1987) was employed in MEGA5 (Tamura et al., 2011), and the bootstrap consensus tree (Felsenstein, 1985) inferred from 1000 replicates is shown in Fig. 3.3.

Fig. 3.4 depicts a consensus bootstrap tree inferred from 1000 replicates obtained from the same data set by using the Maximum Likelihood (ML) method (Hasegawa et al., 1985) that is integrated in MEGA5.

The results of the NJ tree are partially supported by those of the ML tree and show strong intermixing between samples from both sites and from both years, thus indicating that no genetic separation between ticks from these sites has occurred. The samples, however, separated into two sister clades that both contained taxa from both sites and from both years and showed strong bootstrap support.

3.2.2.2 Comparison of borrelia*-positive and -negative ticks from 2006*

No borrelia-positive ticks from 2007 or afterwards were included in the mtMLST scheme for ticks. Therefore a comparison of borrelia-positive and negative ticks had to be restricted to 37 ticks (16 borrelia-positives and 21 borrelia-negatives) from 2006 that were not included in later parts of this study, such as a blood meal analysis (chapter 5) or the prevalence of endobacterial symbionts (chapter 6).

To reconstruct the phylogeny of these samples, both NJ and ML methods were employed through MEGA5, and the consensus bootstrap trees are shown in Figs. 3.5 and 3.6, respectively. While most samples remained intermixed and showed no sign of separation, two clusters of borrelia-negative samples were observed on both trees and with strong bootstrap support (see arrows in Figs. 3.5 and 3.6). These two groupings consisted of three and four ticks, respectively, that had been collected in RW in April 2006 and on two different occasions.

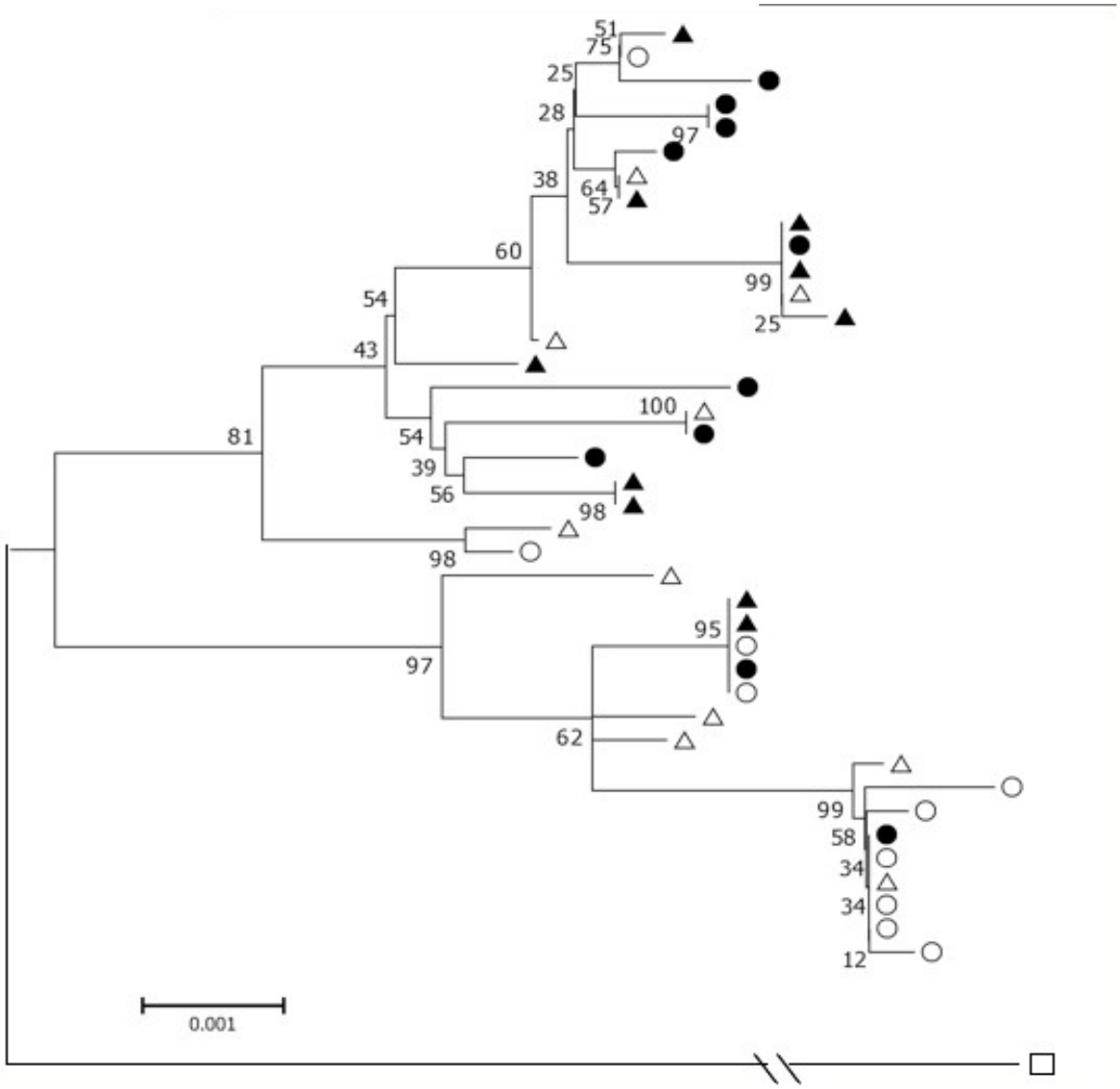


Fig. 3.3. Phylogenetic reconstruction inferred from the concatenated MLST mtDNA sequences of 40 ticks sampled in BW and RW in 2007 and 2008 using the Neighbour-Joining (NJ) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an outgroup (represented by rectangle, □). Ticks from BW are represented by triangles (Δ, ▲), while ticks from RW are depicted as circles (◌, ●). Ticks from 2007 are shown by empty symbols, and ticks from 2008 by filled symbols. The outgroup is not to scale, as indicated by slashes. There was a total of 3195 positions in the final dataset. The scale bar represents 0.1% nucleotide difference. Bootstrap values are shown on the branch nodes.

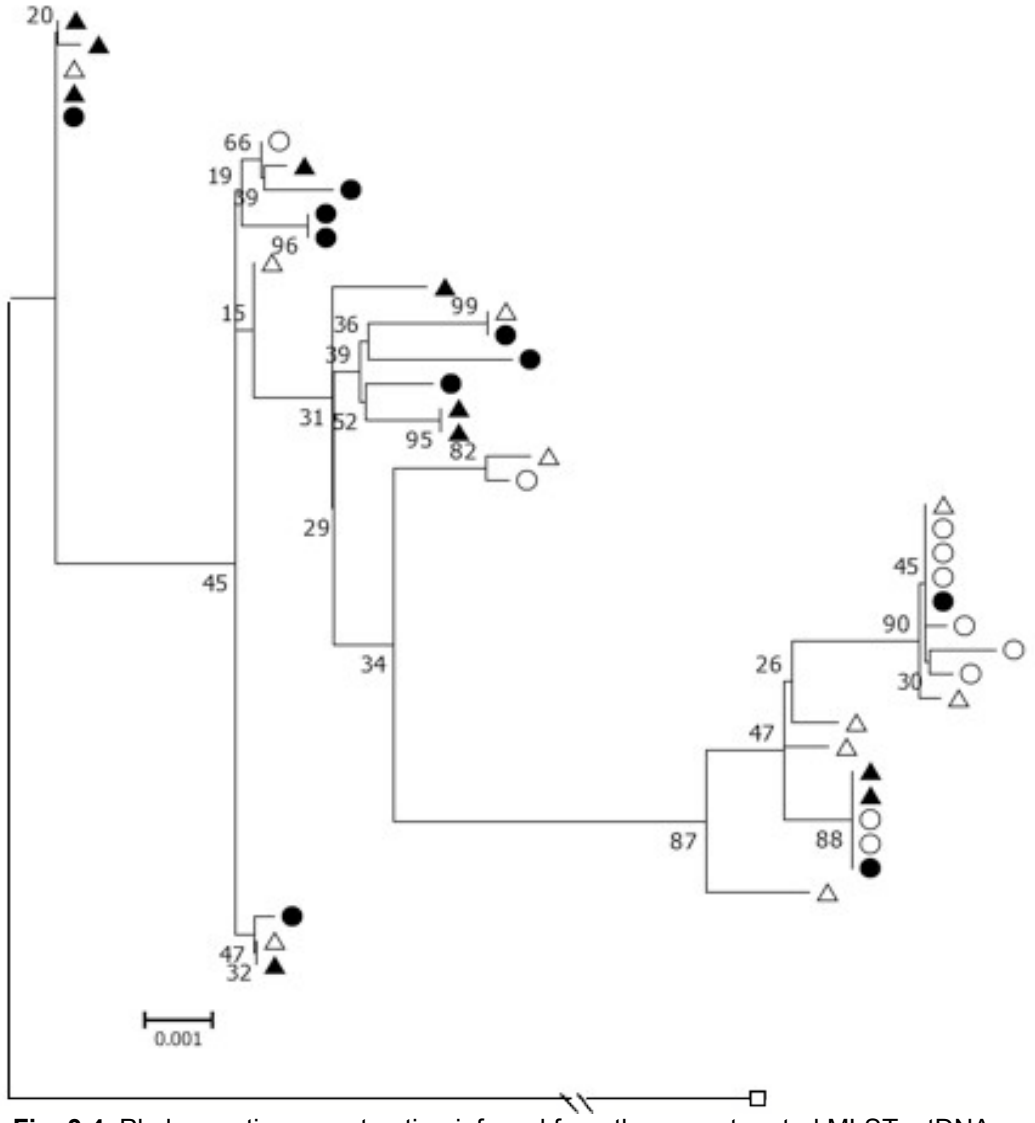


Fig. 3.4. Phylogenetic reconstruction inferred from the concatenated MLST mtDNA sequences of 40 ticks sampled in BW and RW in 2007 and 2008 using the Maximum Likelihood (ML) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an outgroup (represented by rectangle, □). Ticks from BW are represented by triangles (Δ, ▲), while ticks from RW are depicted as circles (◌, ●). Ticks from 2007 are shown by empty symbols, and ticks from 2008 by filled symbols. The outgroup is not to scale, as indicated by slashes. There was a total of 3195 positions in the final dataset. The scale bar represents 0.1% nucleotide difference. Bootstrap values are shown on the branch nodes.

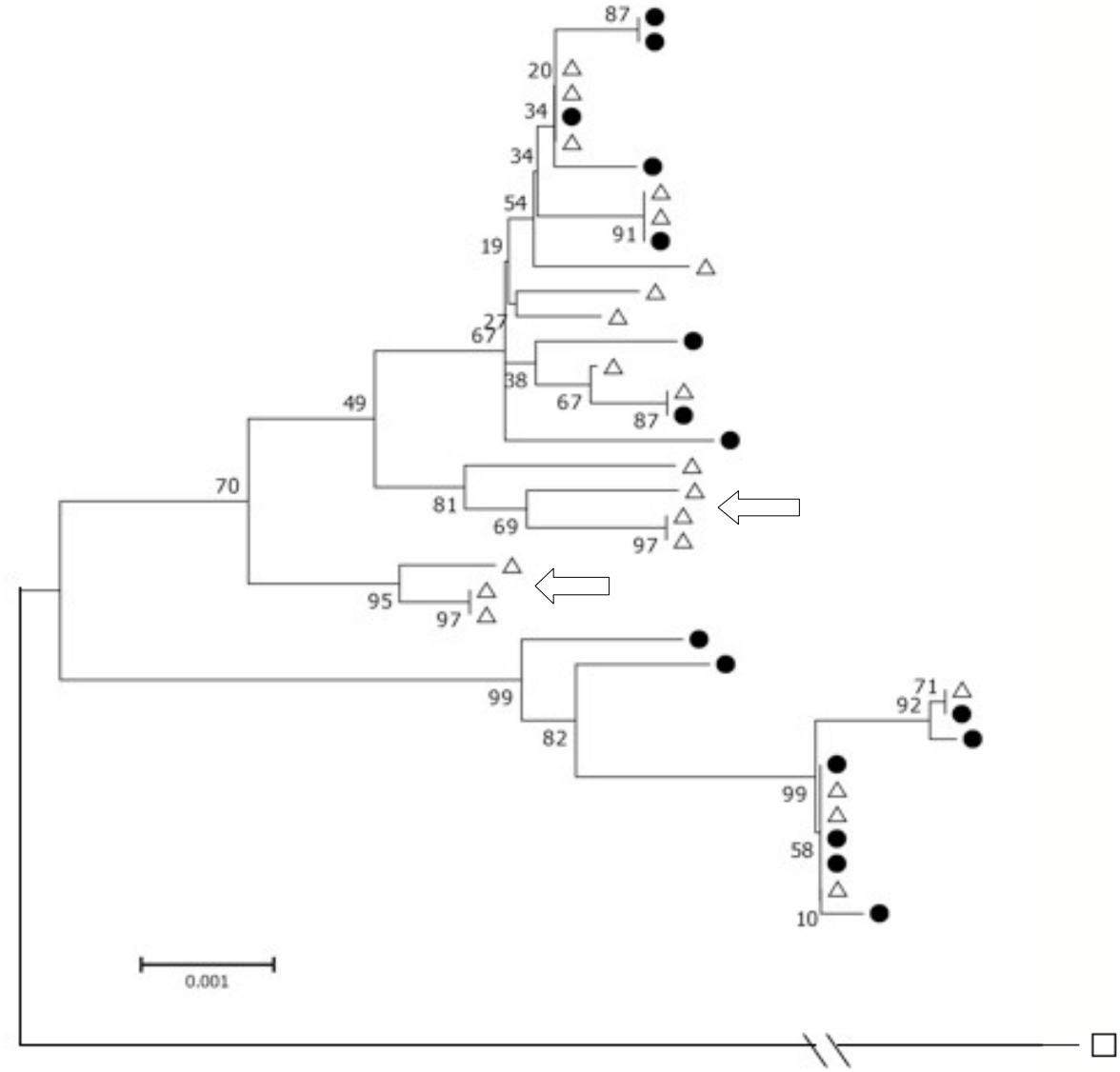


Fig. 3.5. Phylogenetic reconstruction inferred from the concatenated MLST mtDNA sequences of 37 borrelia-positive and negative ticks sampled in BW and RW in 2006 using the Neighbour-Joining (NJ) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an outgroup (represented by rectangle, □). Borrelia-negative samples are shown by empty triangles (Δ), and borrelia-positive ticks by filled circles (●). Arrows indicate clusters of borrelia-negative samples. The outgroup is not to scale, as indicated by slashes. There was a total of 3195 positions in the final dataset. The scale bar represents 0.1% nucleotide difference. Bootstrap values are shown on the branch nodes.

Fig. 3.6. Phylogenetic reconstruction inferred from the concatenated MLST mtDNA sequences of 37 borrelia-positive and negative ticks sampled in BW and RW in 2006 using the Maximum Likelihood (ML) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an outgroup (represented by rectangle, □). Borrelia-negative samples are shown by empty triangles (Δ), and borrelia-positive ticks by filled circles (●). Arrows indicate clusters of borrelia-negative samples. The outgroup is not to scale, as indicated by slashes. There was a total of 3195 positions in the final dataset. The scale bar represents 0.1% nucleotide difference. Bootstrap values are shown on the branch nodes.

3.2.3 Phylogenetic comparison of Latvian and British *I. ricinus* populations

This phylogenetic reconstruction was carried out in order to demonstrate the concept, which has been explored in detail in the PhD thesis of Ruth Dinnis, that *I. ricinus* forms phylogenetically distinct subpopulations across its distribution in Europe that can be differentiated by means of analysing their concatenated mtDNA in a MLST scheme. Therefore 20 samples each from Latvia and from the UK that had been collected in 2007 were randomly selected for a phylogenetic analysis. The resulting consensus bootstrap trees were inferred from their concatenated sequences by using the NJ and ML methods that are shown in Figs. 3.7 and 3.8, respectively. Both methods revealed that the majority of the samples from each country would cluster together in two distinct sister clades, while a small amount of samples from each country could be found in the clade of the other country. The Latvian samples showed a deeper and more complex phylogeny than the British ticks, which were clustered in a more homogenous and shorter clade.

3.2.4 Multiple gene analysis of *I. scapularis* populations from North America

In order to assess the phylogenetic relationship between populations of *I. scapularis* in North America, tick samples collected in several parts of continental USA in 2007 were compared. These consisted of 45 adults from the Northeast (Connecticut and Massachusetts), 52 adults from the Midwestern region (Minnesota and Wisconsin) and 53 adults from Mississippi. It was attempted to emulate the successful mtMLST scheme established for *I. ricinus* populations in Europe by testing the US samples for the same fragments of six mitochondrial genes. The results of the PCR reactions, however, were mixed, with only the primers for 12S, *cox1* and *cox3* delivering successful amplifications for a portion of the samples. No PCR products for *atp6*, *cytb* or *cox2* could be amplified for more than very few samples. Several approaches were attempted to overcome these problems. Different PCR conditions were varied, such as annealing temperature, primer and $MgCl_2$ concentrations, were varied. Different combinations of newly designed primers were tested, including those for *nad4* and *nad5* (see section 3.2.1).

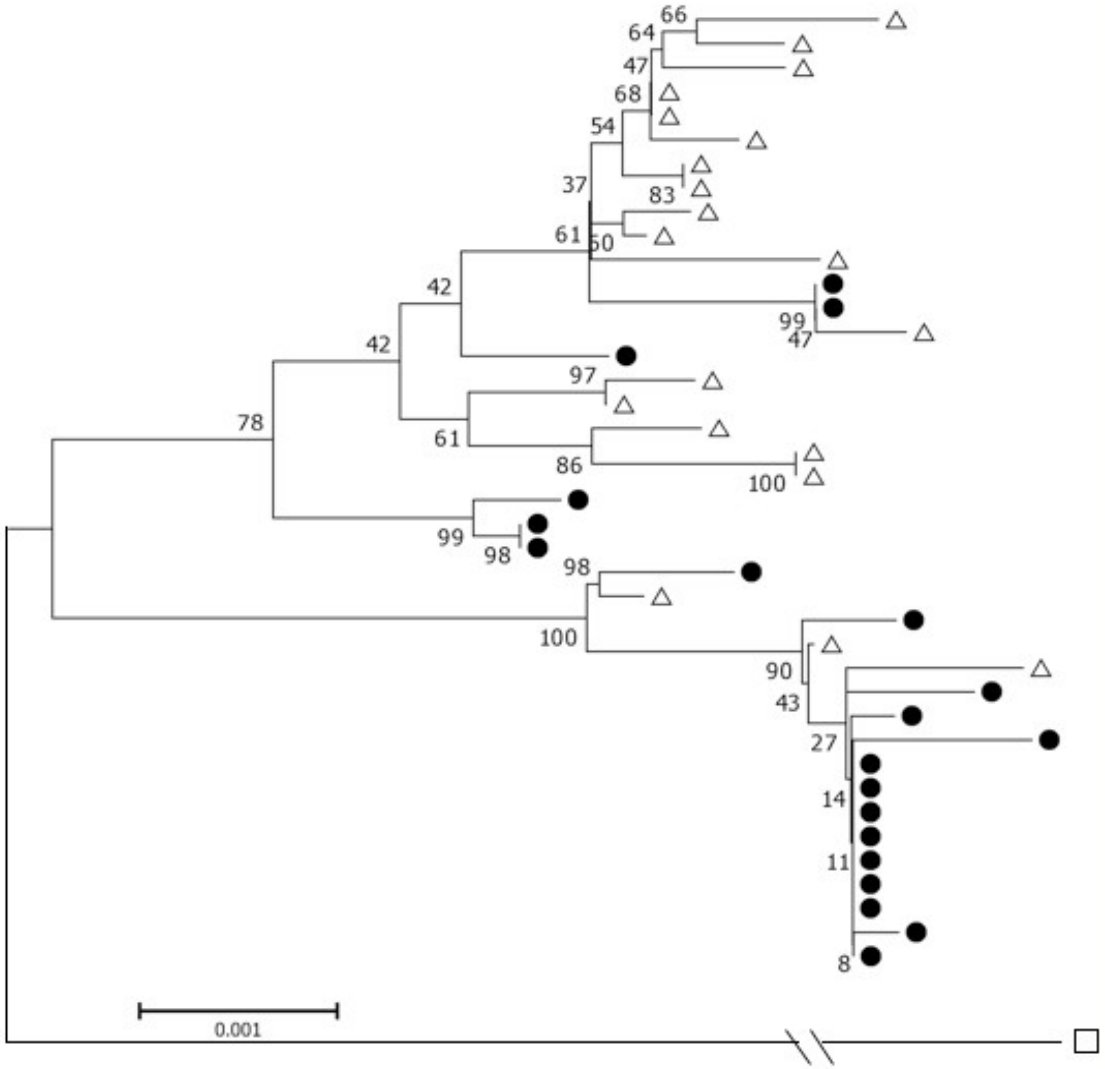


Fig. 3.7. Phylogenetic reconstruction inferred from the concatenated MLST mtDNA sequences of 40 ticks sampled in Latvia and Britain in 2007 using the Neighbour-Joining (NJ) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an outgroup (represented by rectangle, □). Ticks from Latvia are represented by empty triangles (Δ), while ticks from Britain are depicted as filled circles (●). The outgroup is not to scale, as indicated by slashes. There was a total of 3195 positions in the final dataset. The scale bar represents 0.1% nucleotide difference. Bootstrap values are shown on the branch nodes.

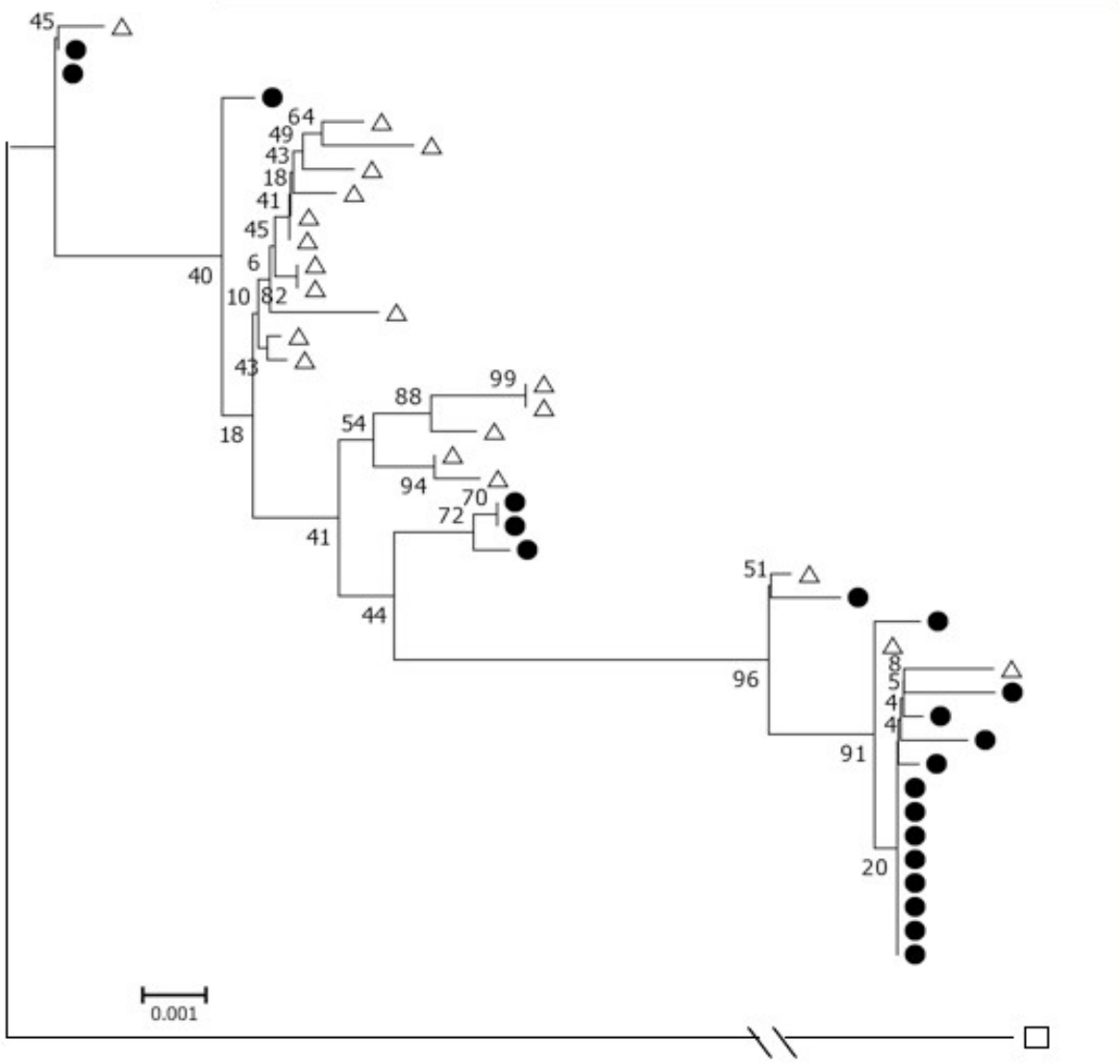


Fig. 3.8. Phylogenetic reconstruction inferred from the concatenated MLST mtDNA sequences of 40 ticks sampled in Latvia and Britain in 2007 using the Maximum Likelihood (ML) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an outgroup (represented by rectangle, □). Ticks from Latvia are represented by empty triangles (Δ), while ticks from Britain are depicted as filled circles (●). The outgroup is not to scale, as indicated by slashes. There was a total of 3195 positions in the final dataset. The scale bar represents 0.1% nucleotide difference. Bootstrap values are shown on the branch nodes.

Ticks from the Northeast and the Midwest yielded more positive signals than ticks from Mississippi, where only the 12S fragment could successfully be amplified. Due to the fact that not all tick samples resulted in positive amplifications for all three gene fragments (12S, *cox1* and *cox3*), the group of ticks for which sequences for these three genes could be obtained was limited to 16 ticks from the Northeast and 12 from the Midwest. A concatenated multi gene sequence of 1677 nucleotides was formed from the three fragment sequences and a phylogenetic hypothesis for these 28 samples was reconstructed. Figure 3.9 shows a phylogenetic tree using the NJ method, while Fig. 3.10 depicts the same set of samples analysed by the ML method. In both trees it was found that samples from a geographical region (Northeast or Midwest) would cluster together, as indicated by the brackets in Figs. 3.9 and 3.10. One group of four samples from the Northeastern region was found in both models to form a sister clade to the remaining samples. This clustering was observed for eight out of 16 samples from the Northeast, and for five out of 12 Midwestern samples in the NJ consensus tree, and for nine out of 16 samples from the Northeast, and for five out of 12 Midwestern samples in the ML tree, respectively. While these clusters showed good bootstrap support in the NJ tree, other branches were less well-supported, especially in the ML tree.

3.3 Discussion

3.3.1 Phylogenetic comparison of *I. ricinus* samples from two sites near Bath, UK

A phylogenetic analysis of ticks collected in 2007 and 2008 in two sites near Bath, UK revealed low levels of nucleotide diversity, which indicates that the ticks form one intermixing population, with no distinction between the two sites or between samples from different years. Since ticks rarely move horizontally over long distances (Eisen & Lane, 2002), the most likely explanation for this intermixing is the distribution of ticks by their hosts. Small rodents normally show low rates of dispersal (Kikkawa, 1964; Gurnell & Hare, 2008) , but other host animals such as birds (Wernham et al., 2002), medium-sized mammals (such as badgers, hedgehogs and hares, see Churchfield, 2008; Gurnell & Hare, 2008) and large mammals (especially deer, see Putman, 2008) are capable of moving regularly between these two sites, which are approximately 2 km apart from each other (see chapter 2, Fig. 2.1), thus allowing the exchange of ticks.

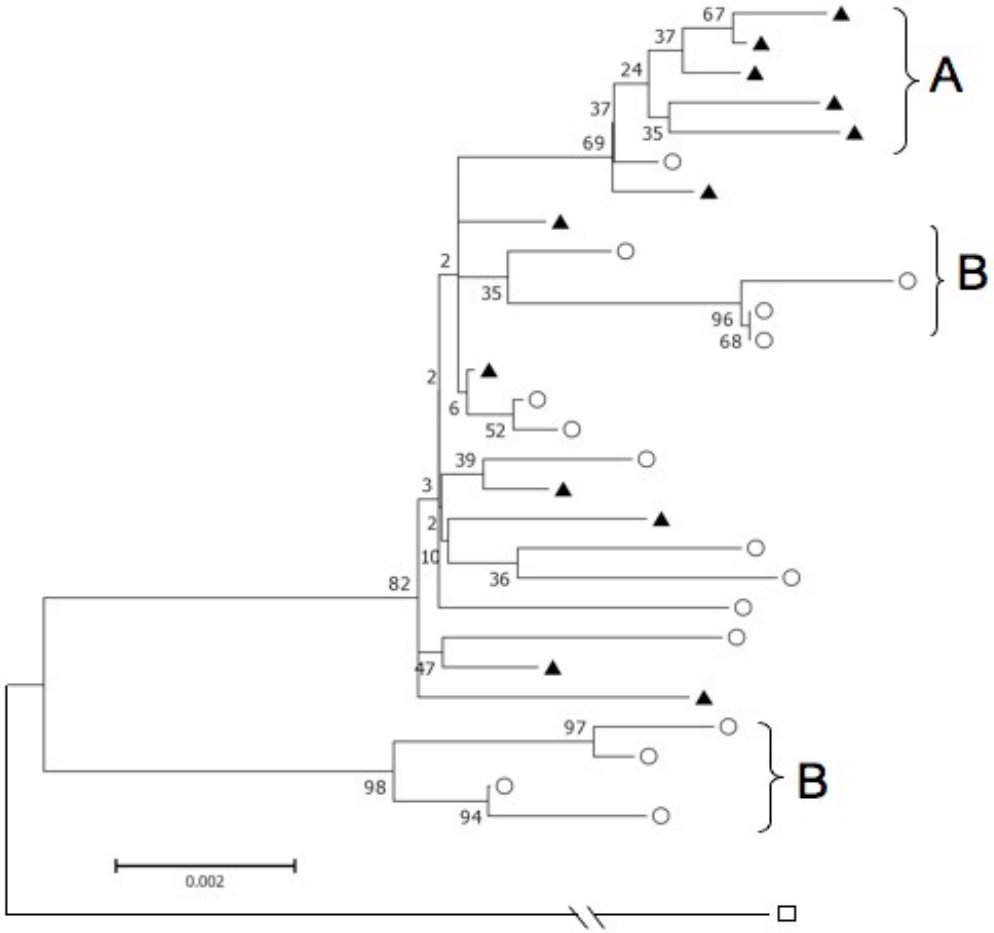


Fig. 3.9. Phylogenetic reconstruction inferred from the concatenated mtDNA sequences of three genes (12S, *cox1* and *cox3*) from 28 ticks sampled in the Northeast (Connecticut and Massachusetts) and Midwest (Minnesota and Wisconsin) of the USA in 2007 using the Neighbour-Joining (NJ) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an out group (represented by rectangle, □). Ticks from the Northeast are represented by empty circles, (◌) while ticks from the Midwestern area are depicted as filled triangles (▲). The out group is not to scale, as indicated by slashes. There was a total of 1677 positions in the final dataset. The scale bar represents 0.2% nucleotide difference. Bootstrap values are shown on the branch nodes. A: cluster of Midwestern taxa, B: clusters of Northeastern taxa.

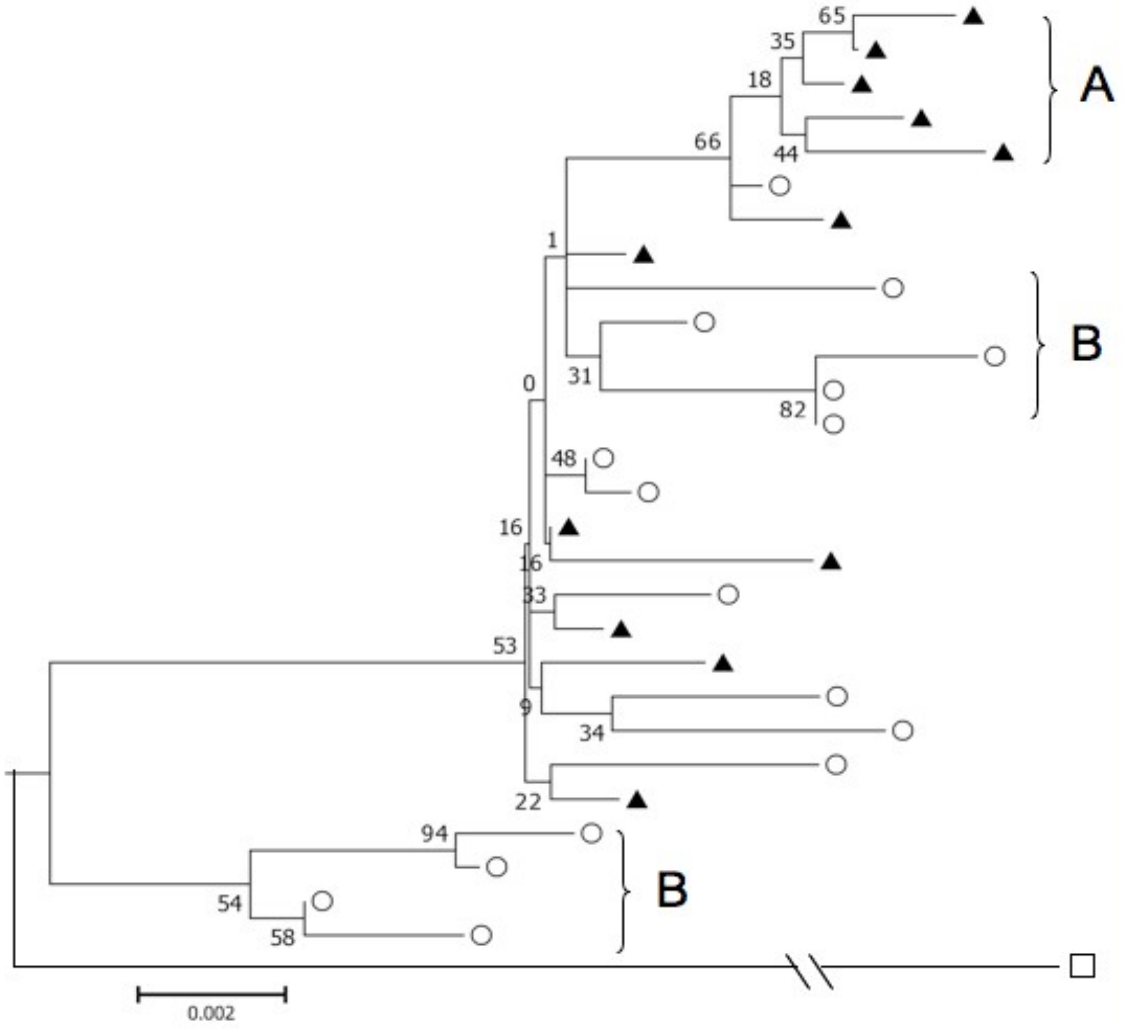


Fig. 3.10. Phylogenetic reconstruction inferred from the concatenated mtDNA sequences of three genes (12S, *cox1* and *cox3*) from 28 ticks sampled in the Northeast (Connecticut and Massachusetts) and Midwest (Minnesota and Wisconsin) of the USA in 2007 using the Maximum Likelihood (ML) method. The bootstrap consensus tree of 1000 repeats is shown. The corresponding mtDNA sequences of *I. persulcatus* were used as an out group (represented by rectangle, □). Ticks from the Northeast are represented by empty circles, (◌) while ticks from the Midwestern area are depicted as filled triangles (▲). The out group is not to scale, as indicated by slashes. There was a total of 1677 positions in the final dataset. The scale bar represents 0.2% nucleotide difference. Bootstrap values are shown on the branch nodes. A: cluster of Midwestern taxa, B: clusters of Northeastern taxa.

The finding that panmixis (unrestricted gene flow within one population, as opposed to clonality, see Kurtenbach et al., 2006) occurred across the area analysed in this study is comparable to a phylogenetic study conducted by Archie & Ezenwa (2011). By analysing two mtDNA gene fragments they could show that generalist nematodes parasitising sympatric host species parasites were completely panmictic across distribution. While the organism used in their study is an endoparasitic nematode with an entirely different biology than an ectoparasitic tick, both can be described as generalists that display a wide range of host preferences.

Unlike in the two later years, ticks collected in 2006 showed partial clustering of borrelia-positive samples (see Figs. 3.5 and 3.6). This might be due to sampling bias, as only a small number of ticks was collected in the two study sites during that year and tested for borrelia prevalence. Therefore it is possible that ticks that were collected at the same time and location had not moved very far from their birth site and were probably siblings. Interestingly, these findings also correlate with observations made in a following chapter on the blood meal analysis of ticks (chapter 5). Here, it was found that borrelia-positive ticks exhibited higher frequencies of positive signals for some groups of host animals.

These results suggest that potential differences between individual ticks or between groups of ticks on such a small geographical scale could be more appropriately assessed by using other phylogenetic markers, such as microsatellites (reviewed by de Meeus et al., 2007) or single nucleotide polymorphisms (SNPs) (reviewed by Zink & Barrowclough, 2008).

3.3.2 Comparison of *I. ricinus* from Latvia and Britain

The comparison between Latvian and British samples revealed clustering of geographically separate samples (see Figs. 3.7 and 3.8). This is a clear demonstration that the mtMLST method developed by our group is capable of differentiating between geographically distinct populations. The two clades remain largely separated, but also show some intermixing, probably due to ticks carried by birds (Ogden et al., 2008; Pietzsch et al., 2008), as migratory passerine birds are known avian hosts for *I. ricinus* (Comstedt et al., 2006). The formation of an *I. ricinus* population on the British Isles occurred most likely in the late phase of the glacial maximum (late GM, ca. 12,000-8,000 years before present, see Mix et al., 2001; Searle et al., 2009), when land released from

retreating ice shields was recolonized by the local fauna, including deer. These act not only as reproductive hosts for *I. ricinus*, but have also been shown to transport ticks over long distances (Ruiz-Fons & Gilbert, 2010; Scharlemann et al., 2008). During the glacial period, separate refugia existed on the Iberian Peninsula and on the Balkans, from where host animals could migrate north in the late GM (reviewed by Emerson & Hewitt, 2005; Miller et al., 2006; Searle et al., 2009). Interestingly, some populations of host species (e.g. the European hedgehog, *Erinaceus europaeus*) originated in different refugia and colonized different parts of Europe, thus potentially giving rise to local clades that could have carried already genetically distinct tick populations with them while moving to new habitats. Following rising sea levels after the pleistocene, the British Isles became separated from the rest of Europe by the North Sea and the English Channel about 8,000-7,500 years ago (Fitch et al., 2005), which led to the formation of a separate British clade of *I. ricinus*. The hypothesis of a recent isolation of this British population would be supported by the phylogenetic clade of British ticks, which showed less divergence than the Latvian samples used in this analysis, which may have originated from a more ancient clade (see Fig. 3.7).

Previous studies (McLain et al., 2001; de Meeus, et al., 2002; Casati et al., 2008; Noureddine et al., 2011) did not observe any phylogeographic structuring between samples from different European and North African locations. Several factors contributed to the absence of correlation between the tick haplotypes and their geographic origin seen in these studies and which deserve a detailed explanation.

The study by McLain et al. (2001) utilised an expansion segment within the *28S* rRNA gene of approximately 150 bp length and analysed six individual *I. ricinus* ticks (among other *Ixodes* species, namely *I. persulcatus*, *I. scapularis*, and *I. pacificus*) that had been collected across the entire East-Western distribution range of the species, including sites in Western Russia, Switzerland and Ireland. It appears likely that the small sample size, combined with a possible lack of phylogenetic resolution obtained form such a short sequence, masked any real phylogeographic structuring, especially in an analysis that attempted to assess interspecific variation among four *Ixodes* species.

Five microsatellite markers were employed by De Meeus et al. (2002) to analyse variations between ticks collected in various sites in Switzerland and in Tunisia. The use of microsatellites can be useful, especially in addition to data from nuclear or mitochondrial

gene sequences. However, it seems probable that the comparison of samples collected within a small area (such as Switzerland) and samples from a geographically distant site, such as Tunisia, might obscure any genetic variation between samples from different Swiss locations that might have become apparent in a finer resolution. In this case the samples from a distant population might be comparable to a phylogeographic outgroup.

The study by Casati et al. (2008) used a sample size of only 26 ticks that had been collected on an approximate North-South transect across Europe, with collection sites ranging from Turku in Southern Finland and Stockholm in Central Sweden to Genoa in Northern Italy. Their analysis employed five different genetic markers, including fragments of three mitochondrial genes (*cox1*, *cox2* and *cytb*), 12S rDNA, and a section of the control region (CR) located between the open reading frames (ORFs) for tRNA-Leu and 12S rDNA, with gene fragment lengths varying between 309 and 1520 bp. It seems likely that the small sample size used in this study included only ticks from a continuous European population of *I. ricinus*, which would exhibit only a low degree of genetic differentiation.

In a recent work by Noureddine et al. (2011), the genetic variability was also compared between ticks from North Africa and samples from various sites in Europe. Here, the authors used a combination of two mitochondrial (16S and *cox1*) and four nuclear (18S, *EF1-α*, *Defensin* and *TROSPA*) gene fragments, resulting in a concatenated sequence of 6,963 bp length. Similar to our own comparison between ticks from BW and RW, these authors also compared both samples on a small scale within a confined study area in Southwestern France as well as samples from sites across the entire distribution range of *I. ricinus*. Unlike our findings, these authors did not observe any structuring in the European population of *I. ricinus*, but could show that the North African clade was highly divergent. They suggested genetic drift following geographic isolation and/or selection pressure due to changed ecological conditions as the main reasons for this divergence. It remains to be debated whether nuclear genes such as *TROSPA* (tick receptor for outer surface protein A [ospA] of *Borrelia* spp., described by Pal et al., 2004) or *defensin*, which is important for the immune system of a tick (Chmelar et al., 2008), underly neutral selection that would make them suitable markers to detect evolutionary changes within a population.The fact that these genes exist in two alleles per individual would also limit their usefulness. The highly divergent North African clade of *I. ricinus* observed by Noureddine et al. (2011) shows interesting parallels to a similarly divergent clade found on the British Isles, as our

findings suggest. Both of these groups have been isolated from a continuous *I. ricinus* population in mainland Europe due to geographical barriers (the Mediterranean Sea and the North Sea/English Channel, respectively) that would limit the exchange of individual ticks with the European population by birds (Ogden et al., 2008; Pietzsch et al., 2008), thereby allowing differentiation due to genetic drift. Noureddine et al. (2011) reported that ecological conditions in the North African habitats led to a shift of questing activity to the winter months, which would also change the pattern of reproduction and thereby reduce the chances of European ticks imported to North Africa to mate with local ticks on a host. The immature stages of the North African clade have also been described to exhibit different host associations than most of their European counterparts and prefer to feed on lizards (eg. *Psammodromus algirus*, Bouattour et al., 2004, cited in Noureddine et al., 2011). This behaviour is comparable to the host preferences observed in immature stages of *I. scapularis* in the Southern clade in the USA (see below). However, it might also be an adaptation to local host fauna, as observed in Portuguese populations of *I. ricinus* (Vitorino et al., 2008). Similar to a study conducted by Oliver et al. (1993b), reciprocal cross-breeding between ticks from the different clades of *I. ricinus* that would produce fertile offspring for several generations could help to establish whether these populations are indeed conspecific or form cryptic species within the *I. ricinus-persulcatus* species complex (Magalhaes et al., 2007; McCoy et al., 2005).

3.3.3 Multi gene analysis of *I. scapularis* samples

In this part of the study it was attempted to apply the successful mtMLST scheme developed for *I. ricinus* populations in Europe to *I. scapularis*, one of the two principal vectors of Lyme Borreliosis in North America (the other being *I. pacificus* in the Western coastal regions of the continent). However, only three of the six gene fragments used in the analysis resulted in sequences from a small set of individual ticks from the Northeastern and Midwestern clades, while no sequences from ticks from the Southern clade could be amplified. The resulting phylogenetic analysis shown in Figs. 3.9 and 3.10 revealed that some ticks from the two different clades would form small separate clusters, while other clades contained samples from both regions. These findings contrast with previous findings obtained by Qiu et al. (2002) and Humphrey et al. (2010), who did not observe genetic structuring within these populations and concluded that they had originated from small founder populations in Southern refugia during the most recent glacial period. The

presence of mixed clusters in our analysis could be explained by the exchange of individual ticks by their host animals, such as deer or migrating birds, as it has also been described for LB spirochaetes (Brinkerhoff et al., 2010; Ogden et al., 2008). The fact that only very few workable 12S sequences, and no sequences for two of the gene fragments employed (*cox1* and *cox3*) could be obtained from any of the 53 ticks collected in Mississippi seem to indicate that tick sequences from the Southern clade are genetically too distinct from the original primers developed from *I. persulcatus* sequences. Geraci et al. (2007) and Ullmann et al. (2005) described intraspecific variation in the genome size of *I. scapularis* as well as the existence of high numbers of multiple gene copies and transposable elements (TE), which would further explain the difficulties to amplify the mitochondrial genes. While previous approaches were made using single or double gene markers such 12S, 16S or 18S (Black et al., 1997; Humphrey et al., 2010; Norris et al., 1996; Qiu et al., 2002; Trout et al., 2009), the development of an mtMLST scheme would not only offer the benefit of a higher genetic resolution thanks to larger concatenated sequences, but would also allow the analysis of different sequence types and their relatedness and patterns of evolutionary descent. The long-awaited publication of the complete genome of *I. scapularis* (Hill & Wikel, 2005; Pagel Van Zee et al., 2007; Ullmann et al., 2003), that should also include mtDNA data, would allow the design of more specific primers for more gene fragments in a full mtMLST scheme.

Two main reasons have emerged why populations of this important vector species should be carefully examined. Populations of *I. scapularis* are expanding both in the Midwestern and Northeastern regions of North America (Dennis et al., 1998; Hamer et al., 2010; Ogden et al., 2006), thereby increasing the risk of surrounding areas to become endemic for LB. Closely monitoring these groups and the ecological conditions in their habitats should help to predict high risk areas for LB and to deliver preventive measures (Diuk-Wasser, 2010). Apart from these public health concerns, the evolutionary history of *I. scapularis* clades has long been debated, with the Northern (or “American”) clade even for some time being assigned the status of a new species, *I. dammini* (Spielman et al., 1979). While the conspecificity of the two clades could be confirmed through reciprocal cross-breeding experiments (Oliver et al., 1993b), the differences in host-association between the Northern and Southern clades have implications for the epidemiology of LB. With immature *I. scapularis* stages in the Southern clade commonly feeding on lizards that

inhibit the transmission of LB spirochaetes (Durden et al., 2002; Oliver et al., 1993a), their transmission potential is much lower than in the Northern regions, where these ticks tend to feed on birds and small mammals, such as white-footed mice and chipmunks, and where LB is hyper-endemic (see Fig. 3.2, reviewed by Kurtenbach et al., 2006; Oliver et al., 1996). Interestingly, a similar preference for reptilian hosts has been recently described by Noureddine et al. (2011) for the immature stages of the North African clade of *I. ricinus* (see section 3.3.2), and for populations in Portugal (Vitorino et al., 2008), which seem to imply convergent adaptations to similar ecological conditions for these two species.

3.4 Conclusions

This chapter demonstrated the establishment of a novel mtMLST scheme for the phylogenetic analysis and molecular typing of *I. ricinus* ticks, as developed by Ruth Dinnis and myself. It was also shown that ticks from two different sites near Bath, UK, exhibited no phylogeographic differentiation, but in a phylogenetic analysis borrelia-positive ticks were found to cluster together. The technique was able to distinguish two distinct clades of ticks from Britain and Latvia, which mirrors their likely evolutionary history. An attempt to replicate this approach with populations of *I. scapularis* from North America did not deliver conclusive results, possibly due to the molecular incompatibility of these ticks with the original primers developed from sequences of another tick species, *I. persulcatus*.

4. Comparative ecology between two tick habitat sites

4.1 Introduction

Ixodes ricinus L., a blood-feeding ectoparasite (Acari: Ixodidae), is the main vector for Lyme borreliosis (LB)-causing spirochaetes in the British Isles (Pietzsch et al. 2005). Lyme borreliosis is an emerging zoonosis that is caused by the bacterium *Borrelia burgdorferi* s.l., Johnson et al. 1984, and is the most common vector-borne disease in the temperate zones of the northern hemisphere (Lindgren & Jaenson 2006). The LB species complex consists of several genospecies with different host associations. *B. afzelii* is predominantly associated with rodent hosts (Hanincova et al. 2003a; reviewed in Kurtenbach et al. 2006) and is more common in most of continental Europe (e.g. Gassner et al. 2010), whereas *B. garinii* and *B. valaisiana* are commonly bird-associated (Hanincova et al. 2003b; reviewed in Kurtenbach et al. 2006). A recent study that analysed borrelia infections in questing ticks from two habitats near Bath, UK, found *B. afzelii* to be entirely absent from Bathampton Woods (BW, see section 2.1.1), while it was present in ticks from Rainbow Woods (RW) (Vollmer et al. 2011). Results taken from this study are summarised in Table 4.1.

Table 4.1. Prevalence of five borrelia genospecies and of mixed infections in nymphs collected in Bathampton Woods (BW) between 2006 and 2008, and in Rainbow Woods in 2008 (RW). Data taken from Vollmer et al., 2011).

Collection site	**BW**			**RW**
Collection year	**2006**	**2007**	**2008**	**2008**
Nymphs screened	184	414	193	82
B. afzelii	0	0	0	2 (2.4%)
B. burgdorferi	0	0	0	0
B. garinii	7 (3.8%)	9 (2.2%)	13 (6.7%)	3 (3.7%)
B. lusitania	0	0	0	0
B. valaisiana	2 (1.6%)	6 (1.4%)	5 (2.6%)	1 (0.7%)
Mixed	1 (0.5%)	2 (0.5%)	6 (3.1%)	3 (3.7%)
Total prevalence	10 (5.4%)	17 (5.2%)	24 (12.4%)	9 (11.0%)

Bathampton Woods can be characterized as woodland habitat, one of the most typical habitats for *I. ricinus* in Britain (reviewed in Pietzsch et al. 2005) and lies on a steep North-east facing slope (see Fig. 2.2), whereas Rainbow Woods is mostly level and shows a more fragmented mix of broadleaved trees, hedges and footpaths that intersect the habitat and separate it from surrounding pastures and fields. It can therefore be regarded as an ecotone,

a habitat type defined as the edges or transition zones between different habitats (reviewed in Despommier et al. 2006). Ecotones are dynamic and complex components of ecological systems and the concept of ecotones can be applied to a broad range of spatial scales, ranging from hedgerows to large-scale vegetation edges that have a massive influence on the surrounding biodiversity (Despommier et al., 2006).

In several studies from North America and Europe, tick densities in ecotonal habitats have been described to be comparatively higher than in surrounding habitats, such as woodlands or pastures (e.g. Lindstrom & Jaenson 2003; Eisen et al. 2004; Daniel & Dusbabek, 1994, cited in Pietzsch et al. 2005). Halos et al. (2010) found that habitat fragmentation resulted in a higher prevalence of *B. burgdorferi* s.l. in questing ticks, possibly due to a higher availability of host animals in the ecotone habitats.

The aims of this chapter are to answer the following questions:

- Are there differences between a woodland and an ecotone habitat in Southwest England with regard to species diversity and densities, tick densities, rodents densities, and tick infestation rates with *I. ricinus*?
- Are there differences between questing ticks sampled in these two habitats in their prevalence of *B. burgdorferi* s.l. infection or their respective borrelia genospecies compositions?

4.2 Results

4.2.1 Assessment of habitat and vegetation types

The assessment of the two habitats near Bath, Bathampton Woods (BW) and Rainbow Woods (RW), showed differences both in plant species diversity and in species abundance. Bathampton Woods contained fewer plant species in the tree and tree under storey/scrub layer than Rainbow Woods. The tree canopy in BW was strongly dominated by yew (*Taxus baccata*) trees, which accounted for about 70%-80%, while they were entirely absent in RW. The under storey in BW consisted mainly of ash (*Fraxinus angustifolia*) trees, holly (*Ilex aquifolium*) and few elderberry (*Sambucus nigra*) bushes. No sycamore (*Acer pseudoplatana*) or birch (*Betula pubescens*) trees were found, and only around 5% of the coverage consisted of beech (*Fagus sylvatica*) and oak (*Quercus robur*) trees, which

were quite common (with estimated coverages of between 6% and 50%, respectively) in RW. In RW, the under storey layer also contained old man's beard (*Clematis vitalba*), rowan (*Sorbus aria*) and about 20% bramble (*Rubus fructicosus*) bushes, while elderberry was missing. The dense canopy of the yew trees in BW limited the availability of light on the forest floor, thereby reducing the number of plant species in the field layer up to 2 m height. The most common species in this layer was hart's-tongue fern (*Asplenium [syn. Phyllitis] scolopendrium*), which accounted for about 40%-50% of the coverage, followed by wild arum (*Arum maculatum*), nightshade (*Circaea lutetiana*) and wood avens (*Geum urbanum*). Other plants, such as common ivy (*Hedera helix*), wood violet (*Viola riviniana*) or false brome (*Brachypodium sylvaticum*) were encountered infrequently, while wild garlic (*Allium ursinum*) was completely absent. It was on the other hand very common in RW, together with ivy, common bracken (*Pteridium aquilinum*), dog's mercury (*Mercurialis perennis*), woodruff (*Galium odoratum*), arum, wall lettuce (*Mycelis muralis*) and violet, which all accounted for about 10%-40% each. The ground layer in BW consisted of moss species (*Eurhynchium* sp., *Fissidens* sp.), which covered a higher proportion of the ground than in RW.

According to the definitions of the British National Vegetation Classification (NVC), both habitats belong to the same vegetation community W12, or *Fagus sylvatica - Mercurialis perennis* woodland, named after its two key species. Within this community, several sub-communities exist, including the *Taxus baccata* sub-community, to which BW belongs, and the more common *Mercurialis perennis* sub-community, as it is found in RW.

The soil types in the two habitats have both been described as lime-rich, leading to beech-dominated woodlands in RW, while BW showed wetter areas due to limited drainage.

Table 4.2 gives a detailed overview of the two sites, describing their vegetation coverage, vegetation communities and soil types.

Table 4.2. Vegetation, plant communities and soil types in two habitats near Bath, UK. BW: Bathampton Woods; RW: Rainbow Woods. Vegetation coverage values: 0: < 1% cover; 1: 1-5%; 2: 6-25%; 3: 26-50%; 4: 51-75%; 5: 76-100% (after Braun-Blanquet, 1928, cited in: van Overbeek et al., 2008). Soil characterisations according to National Soil Resources Institute, Cranfield University, UK (http://www.landis.org.uk/soilscapes/, 2011).

Plant species, height range (m)	Common name	Vegetation coverage values	
		BW	RW
Tree canopy, > 8.0			
Acer pseudoplatana	Sycamore	0	1
Betula pubescens	Down birch	0	2
Fagus sylvatica	Common beech	1	3
Quercus robur	Pedunculate oak	1	2
Taxus baccata	Yew	4	0
Tree under storey/scrub, 2.0 - 8.0			
Clematis vitalba	Old man's beard	0	1
Fraxinus angustifolia	Narrow-leafed ash	1	1
Ilex aquifolium	European holly	2	2
Rubus fructicosus	Brambles	0	2
Sambucus nigra	European elderberry	1	0
Sorbus aria	Rowan	0	1
Field, 0.2 - 2.0			
Allium ursinum	Wild garlic	0	3
Arum maculatum	Wild arum	2	2
Asplenium (syn. Phyllitis) scolopendrium	Hart's-tongue fern	3	1
Brachypodium sylvaticum	False brome	1	1
Circaea lutetiana	Nightshade	2	1
Galium odoratum	Woodruff	1	2
Geum urbanum	Wood avens	2	1
Hedera helix	Common ivy	1	3
Melica uniflora	Wood melick	1	1
Mercurialis perennis	Dog's mercury	1	2
Mycelis muralis	Wall lettuce	1	2
Pteridium aquilinum	Common bracken	1	2
Viola riviniana	Wood violet	1	2
Ground, < 0.2			
Eurhynchium praelongum	Rough-stalked feather-moss	2	2
Fissidens taxifolius	Common feather-moss	3	1

Table 4.2. (continued).

	BW	RW
Vegetation community		
Taxus baccata sub-community of British NVC **W12** *Fagus sylvatica-Mercurialis perennis* woodland	X	
Mercurialis perennis sub-community of British NVC community **W12** *Fagus sylvatica-Mercurialis perennis* woodland		X
Soil type		
Lime-rich loamy and clayey soils with impeded drainage. Base-rich pastures and classic 'chalky boulder clay' ancient woodlands; some wetter areas and lime-rich flush vegetation.	X	
Shallow lime-rich soils over chalk or limestone. Herb-rich downland and limestone pastures; limestone pavements in the uplands; Beech hangers and other lime-rich woodlands.		X

Apart from assessing the vegetation and soil types, the abundance of vertebrate host species was also estimated (see Table 4.3). These estimates were mainly based on visual sightings, but in the case of largely nocturnal species, such as badger (*Meles meles*), hedgehog (*Erinaceus europaeus*) and fox (*Vulpes vulpes*), the surrounding area was screened for signs, including tracks, droppings, burrows and road kill.

The most commonly sighted mammal species were rodents, including grey squirrels (*Sciurus carolinensis*), observed mostly in the tree canopy, wood mice (*Apodemus sylvaticus*), yellow-necked mice (*A. flavicollis*), and bank voles (*Myodes glareolus*) and, occasionally, field voles (*Microtus agrestis*). The latter four species were caught in live traps (see section 4.2.2.5). Rabbits (*Oryctolagus cuniculus*) and roe deer (*Capreolus capreolus*) were seen more frequently in BW than in RW, while more signs of badgers were found in RW. Domestic sheep (*Ovis aries*) were encountered on pasture areas bordering both habitats, but not in the biotopes themselves.

Robins (*Erithacus rubecula*), great tits (*Parus major*) and blackbirds (*Turdus merula*) were the most common bird species in BW, followed by dunnocks (*Prunella modularis*) and blue tits (*Parus caeruleus*). RW showed a higher diversity of bird species, which also included house sparrows (*Passer domesticus*), buzzards (*Buteo buteo*), eurasian jays (*Garrulus glandarius*), wood pigeons (*Columba palumbus*) and thrushes (*T. iliacus, T. philomelos*). Common pheasants (*Phasianus colchicus*) were seen occasionally in both sites.

Common lizards (*Zootoca [syn. Lacerta] vivipara*) were absent in both habitats.

Table 4.3. Occurrence of host species in two habitats near Bath, UK. BW: Bathampton Woods; RW: Rainbow Woods. Host occurrence values: 0: not sighted; +: sighted once; ++: sighted on 2-5 occasions; +++: sighted on almost every occasion. 1): Caught in traps, see section 4.2.2.

Host group/species	Common name	BW	RW
Mammalia	**Mammals**		
Capreolus capreolus	Roe deer	+++	++
Ovis aries	Domestic sheep	++	+
Meles meles	Badger	+	++
Vulpes vulpes	Fox	+	+
Mustela putorius	European polecat	0	0
Mustela erminea	Stoat	0	0
Mustela nivalis[1)]	Weasel	0	+
Oryctolagus cuniculus	Rabbit	0	+
Lepus europaeus	Brown hare	0	0
Sciurus carolinensis	Grey squirrel	+++	+++
Glis (Myoxus) glis	Edible dormouse	0	0
Rattus norvegicus	Common rat	0	0
Myodes glareolus[1)]	Bank vole	+++	+++
Microtus agrestis[1)]	Field vole	+	0
Apodemus sylvaticus[1)]	Wood mouse	+++	+++
Apodemus flavicollis[1)]	Yellow-necked mouse	+	0
Mus musculus	House mouse	0	0
Erinaceus europaeus	Hedgehog	0	+
Talpa europaea	Mole	0	0
Sorex araneus	Common shrew	0	0
Aves	**Birds**		
Buteo buteo	Common buzzard	+	++
Columba palumbus	Wood pigeon	+	++
Passer domesticus	House sparrow	0	++
Phasianus colchicus	Common pheasant	+	+
Erithacus rubecula	Robin	+++	+++
Turdus merula	Common blackbird	+++	+++
Turdus iliacus	Redwing	0	+
Turdus philomelos	Song thrush	+	+
Turdus pilaris	Fieldfare	0	0
Parus major	Great tit	+++	++
Parus caeruleus	Blue tit	+	++
Garrulus glandarius	Eurasian jay	+	++
Fringilla coelebs	Chaffinch	0	+

Table 4.3. (continued).

Host group/species	Common name	BW	RW
Prunella modularis	Dunnock	++	++
Sitta europaea	Eurasian nuthatch	+	+
Troglodytes troglodytes	Winter wren	+	+
Sylvia atricapilla	Blackcap	0	+
Reptilia	**Reptiles**		
Zootoca (Lacerta) vivipara	Common lizard	0	0

4.2.2 Animal trapping

4.2.2.1 Collection of ticks in two habitats near Bath, UK in 2006-2009

Between 2006 and 2009, nymphal and adult ticks were collected in a variety of sites around Bath by members of the Borrelia group of the University of Bath. While BW has consistently been sampled since 2006, RW has only been sampled since 2007, thereby limiting the number of available data from this site. Table 4.4 summarises the findings for both sites, showing the number of occasions ticks were collected, the mean numbers and the range between minimum and maximum values for both nymphal and adult ticks. Temperature and relative humidity were obtained from the website of the Meteorological Office (http://www.metoffice.gov.uk/climate/uk/datasets/) where possible. The presence of larval ticks was recorded, but the larvae were not counted or collected, as they are less relevant for the monitoring of borrelia prevalence than nymphal or adult ticks.

The mean densities both for nymphs and adults in BW showed an increasing trend between March and a peak in June, followed by declining numbers over the later summer and autumn months. The tick densities exhibited a strong variation within each month, ranging for example in the samples taken in June from six to 134 ticks per 100 m^2. The peak in tick densities correlated weakly and non-significantly with the maximum relative humidity of about 80% (Spearman's rank correlation; $r^2 = 0.226757$, $P > 0.05$), which declined afterwards to values of 60-70%.

Tick densities in RW reached a maximum of 75/100 m^2 in March and remained at about 50 per 100 m^2 throughout spring before declining sharply during the summer months. A later increase could be seen during late summer and autumn. No significant differences in the mean values between BW and RW were found (Mann-Whitney *U* test, $U = 31$, $P > 0.05$).

Table 4.4: Mean densities of *I. ricinus* nymphs and adults collected in (A) Bathampton Woods (BW), and (B) Rainbow Woods (RW), between 2006 and 2009. Numbers in brackets indicate minimum and maximum values. N+A: Nymphs and Adults; RH: Relative humidity.

(A):

Month	Sampling days	Total ticks (N+A)/ 100 m^2	Nymphs/ 100 m^2	Adults/ 100 m^2	Tempera-ture (°C)	RH (%)
March	**2**	48.5 (25-72)	45.5 (23-68)	3.0 (2-4)	15.0 (14-16)	65.5 (54-77)
April	**6**	35.3 (3-74)	32.3 (7-70)	3.0 (1-5)	16.8 (13-23)	62.5 (47-75)
May	**6**	48.0 (16-86)	42.3 (14-82)	5.7 (2-12)	15.7 (10-22)	73.8 (58-92)
June	**3**	84.0 (6-134)	78.3 (4-121)	5.7 (2-13)	16.0 (15-17)	80.0 (76-84)
July	**-**	NA	NA	NA	NA	NA
August	**6**	20.6 (8-35)	20.2 (8-33)	0.4 (0-2)	19.0 (16-22)	57.5 (43-66)
September	**3**	13.0 (3-20)	11.7 (3-19)	1.3 (0-3)	18.3 (18-19)	70.0 (65-78)
October	**1**	6.0 ()	6.0 ()	0 ()	13.0 ()	65.0 ()
November	**1**	29.0 ()	28.0 ()	1.0 (1)	16.0 ()	71.0 ()
Total	**28**					

(B):

Month	Sampling days	Total ticks (N+A)/ 100 m^2	Nymphs/ 100 m^2	Adults/ 100 m^2	Tempera-ture (°C)	RH (%)
March	**1**	75.0 ()	73.0 ()	2.0 ()	7.2 ()	NA
April	**5**	41.2 (13-56)	41.2 (13-56)	0 (0)	14.0 (11-17)	60.8 (58-65)
May	**3**	58.7 (42-84)	51.3 (30-84)	7.3 (0-20)	17.0 (12-22)	60.0 (58-64)
June	**1**	12.0 ()	10.0 ()	2.0 ()	13.6 ()	NA
July	**1**	7.0 ()	4.0 ()	3.0 ()	15.4 ()	NA
August	**4**	23.7 (21-24)	20.0 (17-22)	3.7 (0-7)	18.7 (17-21)	63.0 (61-65)
September	**2**	32.0 (26-38)	28.5 (23-34)	3.5 (3-4)	21.0 (19-23)	59.0 (55-63)
October	**1**	31.0 ()	30.0 ()	1 ()	12.0 ()	66.0 ()
November	**-**	NA	NA	NA	NA	NA
Total	**18**					

4.2.2.2 Collection of ticks in two habitats near Bath, UK in 2010

Ticks were collected after each completed session of rodent trapping (see section 4.2.2.4 below). The temperature and RH were recorded during the tick collections by using a handheld electronic multisensor, thereby obtaining more accurate and localised data than the meteorological records used previously. A total number of 385 ticks were collected in BW, and 70 were collected in RW. The results are shown in Table 4.5.

The findings for BW show that densities for both nymphal and adult ticks reached a

maximum of 125 ticks/100 m^2 in May, followed by a steep decline during the summer and early autumn. A negative trend between tick density and temperature (Spearman's rank correlation; $r^2 = 0.045918$, $P > 0.05$) and a positive trend between tick density and RH could be found (Spearman's rank correlation; $r^2 = 0.031888$, $P > 0.05$). It should be noted, however, that the RH values measured in 2010 were generally higher than those previously recorded.

Tick densities in RW were significantly lower than in BW (Mann-Whitney *U* test, $U = 48$, $P < 0.05$), with a relative maximum of only 24 ticks/100 m^2 found in April and 18 ticks/100 m^2 in May. Extremely low densities were observed during the summer months, while a small increase could be seen in the autumn. Again, a negative trend between tick density and temperature was observed (Spearman's rank correlation; $r^2 = 0.292208$, $P > 0.05$), while a positive trend between tick density and RH was found (Spearman's rank correlation; $r^2 = 0.020408$, $P > 0.05$). The RH showed a higher degree of variation than in BW, and was again higher than previously recorded values.

Table 4.5: Number of *I. ricinus* nymphs and adults collected in (A) Bathampton Woods (BW) and (B) Rainbow Woods (RW) in 2010. N+A: Nymphs and Adults; RH: Relative humidity.

(A):

Month	Ticks (N+A)/ 100 m^2	Nymphs/ 100 m^2	Adults/ 100 m^2	Temperature (°C)	RH (%)
March	31	22	9	8.5	71.9
April	44	40	4	13.7	81.2
May	125	118	7	12.3	83.3
June	93	86	7	14.2	71.6
July	37	36	1	17.1	82.4
August	36	34	2	15.7	85.8
September	19	18	1	15.0	77.1
October	NA	NA	NA	NA	NA
Median	**37**	**36**	**4**	**14.1**	**81.2**

(B):

Month	Ticks (N+A)/ 100 m^2	Nymphs/ 100 m^2	Adults/ 100 m^2	Temperature (°C)	RH (%)
March	NA	NA	NA	NA	NA
April	24	18	6	12.4	91.3
May	18	15	3	14.8	73.6
June	4	4	0	12.4	85.0
July	2	0	2	18.2	76.7
August	0	0	0	17.2	84.4
September	7	6	1	17.4	84.6
October	15	14	1	12.1	80.5
Median	**7**	**6**	**1**	**14.8**	**84.4**

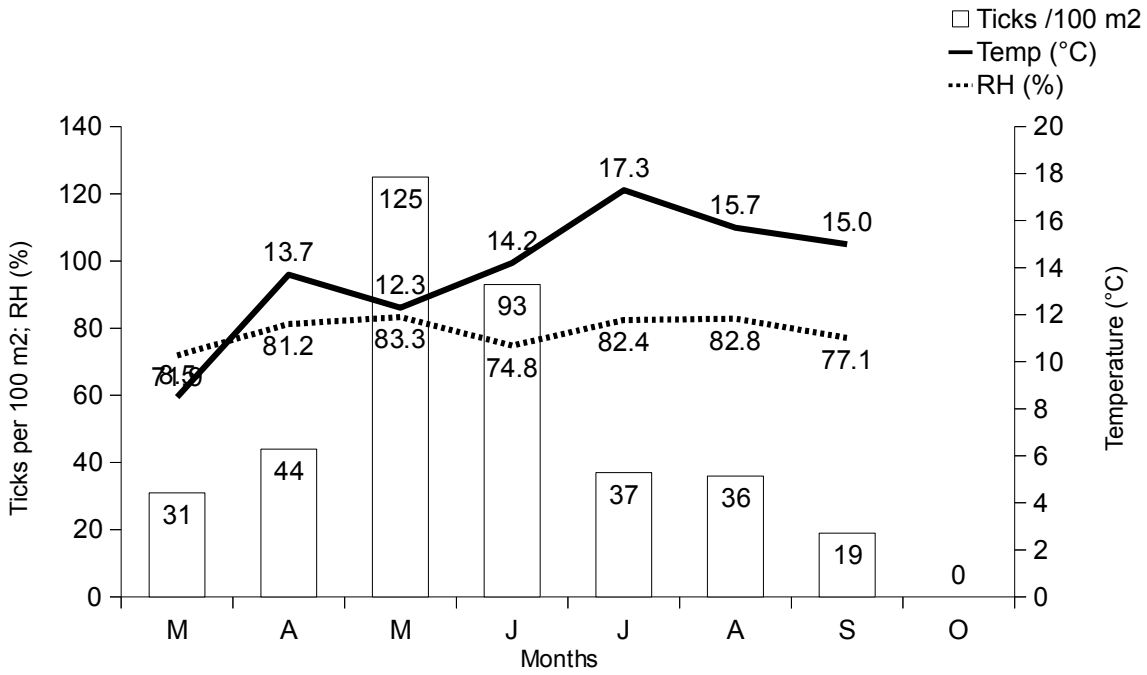


Fig. 4.1: Median tick densities (nymphs and adults), temperature and relative humidity (RH) observed in Bathampton Woods, between March and October 2010.

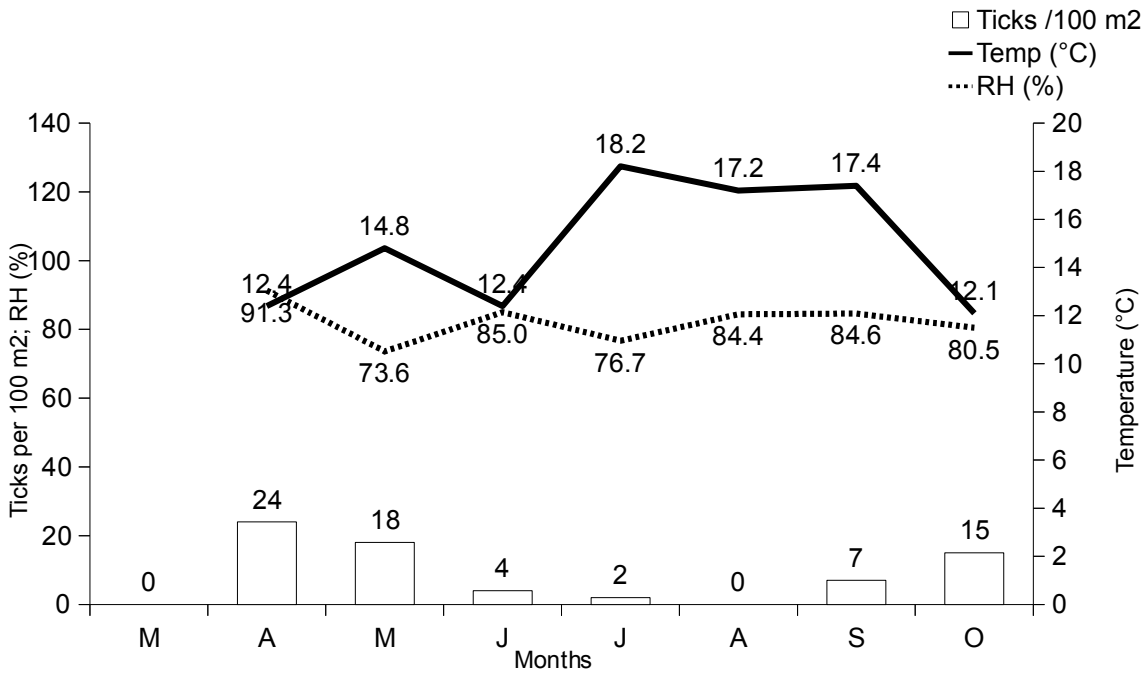


Fig. 4.2: Median tick densities (nymphs and adults), temperature and relative humidity (RH) observed in Rainbow Woods, between April and October 2010.

4.2.2.3 Bird trapping in Bratton 2009/2010

Between August 2009 and June 2010, a total number of 1585 birds (belonging to nine different species) were caught and examined in a ringing station in Bratton, West Somerset. Of these, 50 (3.2%) were infested with ticks of all three stages which belonged to four different *Ixodes* species (see Table 4.6). The most common tick species was *I. ricinus*, of which 24 (one larva, 14 nymphs, nine adults) specimen were found to parasitise on birds from all nine species. This resulted in overall infestation rates of 0.06% for larvae, 0.88% for nymphs and 0.57% for adults. Two other ticks species, *I. arboricola* and *I. frontalis*, accounted for about a quarter of all infestations each, with *I. arboricola* equally divided between nymphs and adults, while almost all *I. frontalis* were adults. One nymphal *I. trianguliceps* was found on a greenfinch (*Carduelis chloris*). Three more larvae could not be identified because their mouthparts were missing.

While the highest absolute number of ticks was found on blue tits (*Parus caeruleus*), the highest percentage of infested birds was observed for greenfinches with 11.9%, followed by blackcaps (*Sylvia atricapilla*, 6.7%) and great tits (*Parus major*, 6.3%) (see Table 4.6). The lowest prevalence of ticks was seen on siskins, of which only two in 384 (0.5%) were infested.

Table 4.6: Infestation rates of ringed birds with four different *Ixodes* tick species, captured between August 2009 and June 2010 in Bratton, West Somerset. L: larvae; N: nymphs; A: adults. 1): Excluding three unidentified larvae.

Bird species	*I. arboricola*			*I. frontalis*			*I. ricinus*			*I. trianguliceps*			Total no. of ticks	Proportion of infested birds
	L	N	A	L	N	A	L	N	A	L	N	A		
Carduelis carduelis (Goldfinch)									1				**1**	**1/29 (3.4%)**
Carduelis chloris (Greenfinch)						3		4			1		**8**	**8/67 (11.9%)**
Carduelis spinus (Siskin)								2					**2**	**2/384 (0.5%)**
Erithacus rubecula (Robin)								2					**2**	**2/52 (3.8%)**
Parus caeruleus (Blue tit)		6	6		1	3		1	1				**18**	**18/672 (2.7%)**
Parus major (Great tit)			1			2	1	2	1				**7**	**7/112 (6.3%)**
Passer domesticus (House sparrow)						3		1	6				**10**	**10/199 (5.0%)**
Sylvia atricapilla (Blackcap)								1					**1**	**1/15 (6.7%)**
Turdus merula (Blackbird)								1					**1**	**1/55 (1.8%)**
Total	**13 (26%)**			**12 (24%)**			**24 (48%)**			**1 (2%)**			**50**[1)]	**50/1585 (3.2%)**

4.2.2.4 Rodent trapping

- **Rodent densities in 2009 and 2010:**

Rodents were captured and examined for tick infestations between July and October 2009 and between April and October 2010. In order to standardise the trapping results and to take numbers of used traps and of trap nights per session into account, values for 100 trapping nights were calculated. For example, a trapping session of four nights and with 25 traps would result in 100 trap nights. This would also allow an estimation of the spatial densities, as 25 traps set about 5 metres apart in a rectangular grid would cover an area of

approximately 400 m^2.

Apart from the two sites described in detail, a third site located on the main campus of the University of Bath was initially used as well, but had to be abandoned due to repeated disturbances of the traps (data not shown).

In 2009, a total of 17 rodents were captured in BW during six nights. These included 13 wood mice (*A. sylvaticus*), of which four were immature males (< 18.0 g body weight), five adult females and four adult males; two adult (1 ♀, 1 ♂) bank voles (*My. glareolus*), and one adult female yellow-necked mouse (*A. flavicollis*) and male field vole (*Mi. arvalis*) each. In RW, 13 animals were caught during four nights, consisting of five wood mice (all adults, 3 ♀, 2 ♂) and eight bank voles (seven adults and one immature, < 17.0 g body weight, all males). No animals were recaptured in either of the two habitats. While the total number of animals caught in BW was higher than in RW, the density of 11.34 animals per 100 trap nights was lower than 13 animals per 100 trap nights found in RW (see Table 4.7A).

Trapping in 2010 in BW resulted in 50 rodents (31 wood mice and 19 bank voles) being caught during a total of 12 nights, of which 30 (20 wood mice, 10 bank voles) were recaptured. Two of the remaining 11 wood mice (plus 4 ♀, 5 ♂ adults) and two of the nine bank voles (plus 2 ♀, 5 ♂ adults) were immature males each. This led to a true density (the difference between initial captures and recaptures) of 6.67 animals (wood mice: 3.66, bank voles: 3.00) per 100 trap nights. Thirty-eight rodents were caught in RW, consisting of 21 wood mice and 17 bank voles. Of these, eight wood mice and six bank voles were recaptured. The remaining 13 wood mice comprised four immature males, two adult females and seven adult males; the 11 bank voles consisted of two immature and nine adult males. The calculated true densities were therefore 8.73 rodents per 100 trap nights, comprising 4.73 wood mice and 4.00 bank voles, respectively (see Table 4.7B).

Spatial densities per 100 m^2 were calculated by dividing the true densities by the factor 4 (to account for a total area of 400 m^2 covered). It was found that RW showed higher overall spatial densities in both 2009 and in 2010, and also higher densities for both wood mice and bank voles in both habitats, with the the exception of wood mice densities in BW in 2009. A general decrease in spatial densities could be seen between 2009 and 2010 (see Table 4.7 A, B).

In addition to the four rodent species targeted in the traps, one male European weasel (*Mustela nivalis*) was found dead in a trap in RW in August 2010. No ticks were found on the animal.

Table 4.7: Densities of four rodent species trapped in BW and RW, (A) between July and October 2009, and (B) between April and October 2010, per 100 trap nights, and per 100 m^2. Numbers in brackets indicate the absolute number of animals caught, per trap nights x 100.
(A):

Rodent species	BW				RW			
	Total captures	**Recap-tures**	**True density**	**Per 100 m^2**	**Total captures**	**Recap-tures**	**True density**	**Per 100 m^2**
A. sylvaticus	8.67 (13/1.5)	0	8.67 (13/1.5)	2.17	5.00 (5/1)	0	5.00 (5/1)	1.25
My. glareolus	1.33 (2/1.5)	0	1.33 (2/1.5)	0.33	8.00 (8/1)	0	8.00 (8/1)	2.00
A. flavicollis	0.67 (1/1.5)	0	0.67 (1/1.5)	0.17	0	0	0	0
Mi. arvalis	0.67 (1/1.5)	0	0.67 (1/1.5)	0.17	0	0	0	0
Total	**11.34 (17/1.5)**	**0**	**11.34 (17/1.5)**	**2.84**	**13.00 (13/1)**	**0**	**13.00 (13/1)**	**3.25**

(B):

Rodent species	BW				RW			
	Total captures	**Recap-tures**	**True density**	**Per 100 m^2**	**Total captures**	**Recap-tures**	**True density**	**Per 100 m^2**
A. sylvaticus	10.33 (31/3)	6.67 (20/3)	3.66 (11/3)	0.92	7.64 (21/2.75)	2.91 (8/2.75)	4.73 (13/2.75)	1.18
My. glareolus	6.33 (19/3)	3.33 (10/3)	3.00 (9/3)	0.75	6.18 (17/2.75)	2.18 (6/2.75)	4.00 (11/2.75)	1.00
A. flavicollis	0	0	0	0	0	0	0	0
Mi. arvalis	0	0	0	0	0	0	0	0
Total	**16.67 (50/3)**	**10.00 (30/3)**	**6.67 (20/3)**	**1.67**	**13.82 (38/2.75)**	**5.09 (14/2.75)**	**8.73 (24/2.75)**	**2.18**

- **Monthly distributions:**

As seen in Fig. 4.3, August was the only month during the trapping period in 2009 in which both wood mice and bank voles were caught in both habitats, and the only month with rodent captures in BW. It also marked the peak density of wood mice caught in BW, while fewer animals were found in this habitat in June and in October, and none at all in September. One female yellow-necked mouse was caught in BW in August 2009 and one male field vole was captured in BW in October 2009 (not shown).

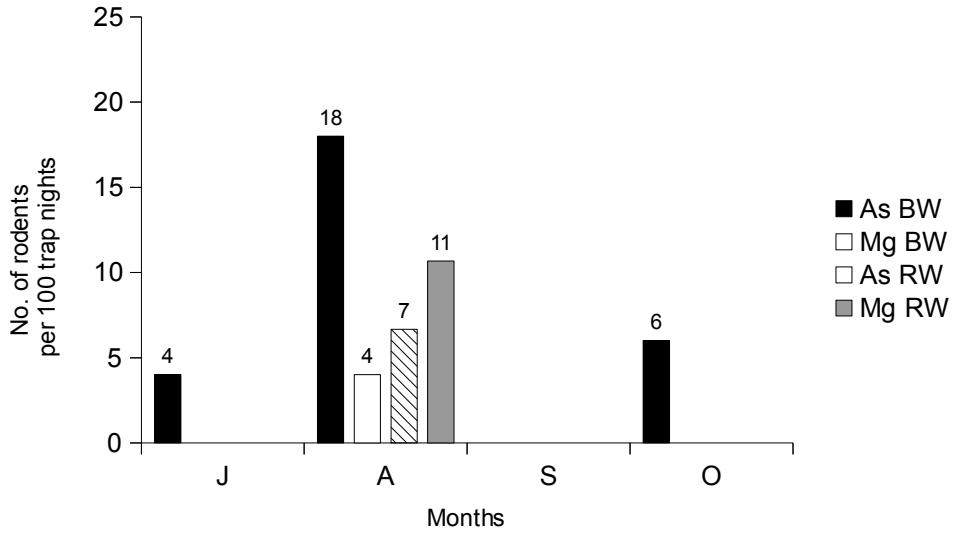


Fig. 4.3: Monthly distribution of rodent densities per 100 trap nights, between July and October 2009. As: *A. sylvaticus*; Mg: *M. glareolus*; BW: Bathampton Woods; RW: Rainbow Woods.

Trappings of wood mice in 2010 showed a bimodal pattern in BW, with peaks in June and in September, while no wood mice were caught in RW before June, after which a steady decline in densities until October could be observed. Bank vole densities in BW exhibited a relative maximum of 12 animals per 100 trap nights in July, whereas a similar maximum density was reached in RW in August (see Fig. 4.4).

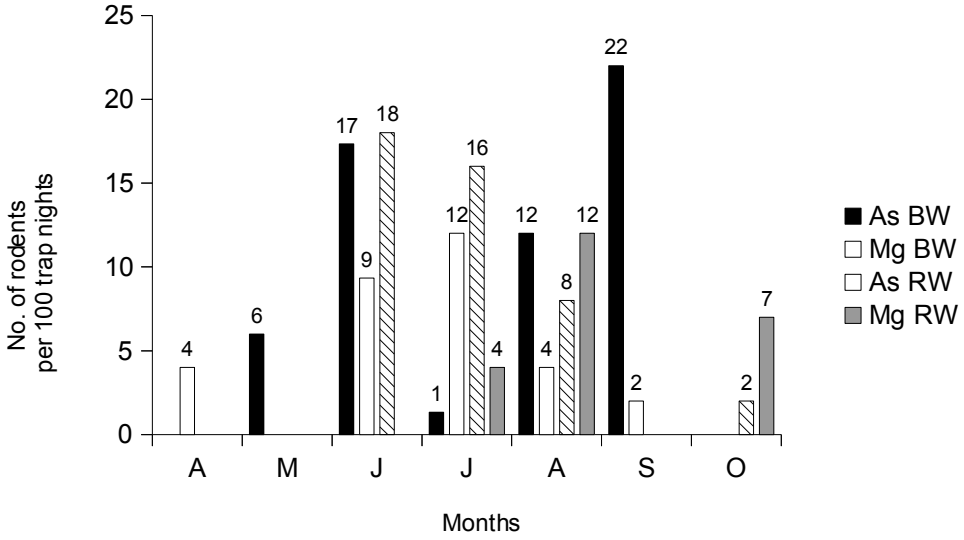


Fig. 4.4: Monthly distribution of rodent densities per 100 trap nights, between April and October 2010. As: *A. sylvaticus*; Mg: *M. glareolus*; BW: Bathampton Woods; RW: Rainbow Woods.

- **Tick infestations:**

Table 4.8 shows that two mature wood mice (or 1.33 rodents with larvae per 100 nights)

in BW were parasitised by *I. ricinus* larvae and were the only rodents found to be infested with ticks in 2009. The rate of infestation increased in 2010, with eight wood mice (including one immature, 2.67 per 100 nights) with larvae feeding on them in BW. In RW, four wood mice (including one immature) were parasitised by larvae (1.45 per 100 nights) and one gravid female by a nymph (0.36 per 100 nights), while two adult bank voles (0.73 per 100 nights) were also found to be infested with larvae.

Table 4.8: Intensity of *I. ricinus* infestations in BW and RW in (A) 2009 and (B) 2010. Figures show amount of rodents with ticks per 100 trap nights. L: larvae, N: nymphs, A: adults.

(A):

Rodent species	BW (L, N, A)	RW (L, N, A)	Total (L, N, A)
A. sylvaticus	1.33 (1.33, 0, 0)	0 (0, 0, 0)	**1.33 (1.33, 0, 0)**
A. flavicollis	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**
My. glareolus	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**
Mi. arvalis	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**
Total	**1.33 (1.33, 0, 0)**	**0 (0, 0, 0)**	**1.33 (1.33, 0, 0)**

(B):

Rodent species	BW (L, N, A)	RW (L, N, A)	Total (L, N, A)
A. sylvaticus	2.67 (2.67, 0, 0)	2.18 (1.45, 0.36, 0)	**4.85 (4.12, 0.36, 0)**
A. flavicollis	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**
My. glareolus	0 (0, 0, 0)	0.73 (0.73, 0, 0)	**0.73 (0.73, 0, 0)**
Mi. arvalis	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**
Total	**2.67 (2.67, 0, 0)**	**2.91 (2.18, 0.36, 0)**	**5.58 (4.85, 0.36, 0)**

While the data above show the rate of animals infested with ticks, they do not take into account the number of ticks feeding on one rodent when captured. Table 4.9 shows the extent of infestations with *I. ricinus*. As seen above, two wood mice in BW that were parasitised by two larvae each were the only rodents found to be infested in 2009, leading to a ratio of 2.00 larvae per infested wood mouse, and an overall infestation rate of 23.53% (Table 4.9A).

In 2010, a total number of 20 larvae (or 6.67 per 100 trap nights) were observed on wood mice captured in BW, resulting in a density of 2.50 larvae per infested wood mouse per 100 trap nights, or an overall infestation rate of 40.0% (20 larvae infesting 50 wood mice). The highest number of ticks were found on immature mice, ranging from two to five larvae per infested animal, while adult mice were infested with between one and three larvae. No other tick stages were encountered, and none of the other rodent species was

found to be infested. In RW, nine larvae and one nymph (3.64 per 100 nights) parasitised five wood mice, which led to a ratio of 1.67 ticks (1.46 larvae and 0.17 nymphs) per infested wood mouse per 100 nights, and overall infestation rates of 42.86% (nine larvae infesting 21 wood mice) and 4.76% (one nymph per 21 mice), respectively. Here, the one infested immature mouse was infested with one larva. Furthermore, two bank voles were infested with one larva each, thus leading to a ratio of 1.00 larvae per bank vole per 100 nights (see Table 4.9 B), and an overall rate of 11.76% (two larvae infesting 17 bank voles). The total numbers of ticks, the extent of their infestation rates and the ratios of ticks per infested rodents per 100 nights increased from 2009 to 2010.

Table 4.9: Extent of *I. ricinus* infestations in BW and RW in (A) 2009 and (B) 2010. Figures show total number of ticks on infested rodents per 100 trap nights, and ratio of ticks per infested rodents per 100 trapping nights. L: larvae, N: nymphs, A: adults.

(A):

Rodent species	BW		RW		Total (L, N, A)	Per infested rodents total
	Total no. ticks (L, N, A)	Per infested rodents	Total no. ticks (L, N, A)	Per infested rodents		
A. sylvaticus	2.67 (2.67, 0, 0)	2.00 (2.00, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	**2.67 (2.67, 0, 0)**	**2.00 (2.00, 0, 0)**
A. flavicollis	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**	**0 (0, 0, 0)**
My. glareolus	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**	**0 (0, 0, 0)**
Mi. arvalis	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**	**0 (0, 0, 0)**
Total	**2.67 (2.67, 0, 0)**	**2.00 (2.00, 0, 0)**	**0 (0, 0, 0)**	**0 (0, 0, 0)**	**2.67 (2.67, 0, 0)**	**2.00 (2.00, 0, 0)**

(B):

Rodent species	BW		RW		Total (L, N, A)	Per infested rodents total
	Total no. ticks (L, N, A)	Per infested rodents	Total no. ticks (L, N, A)	Per infested rodents		
A. sylvaticus	6.67 (6.67, 0, 0)	2.50 (2.50, 0, 0)	3.64 (2.91, 0.36, 0)	1.67 (0.46, 0.17, 0)	**10.31 (9.58, 0.36, 0)**	**4.17 (2.96, 0.17, 0)**
A. flavicollis	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**	**0 (0, 0, 0)**
My. glareolus	0 (0, 0, 0)	0 (0, 0, 0)	0.73 (0.73, 0, 0)	1.00 (1.00, 0, 0)	**0.73 (0.73, 0, 0)**	**1.00 (1.00, 0, 0)**
Mi. arvalis	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)	**0 (0, 0, 0)**	**0 (0, 0, 0)**
Total	**6.67 (6.67, 0, 0)**	**2.50 (2.50, 0, 0)**	**4.37 (3.64, 0.36, 0)**	**2.67 (1.46, 0.17, 0)**	**11.04 (10.31, 0.36, 0)**	**5.17 (3.96, 0.17, 0)**

Table 4.10 shows the monthly distribution of ticks collected from rodents in 2010. The data for both sites matched those for rodent densities (see Fig. 4.4). Both the highest number of ticks and the highest extent of infestation in BW occurred in June, which coincided with the peak in rodent densities. A second smaller peak both in tick numbers and density per rodent was observed in September, again similar to the smaller peak in rodent densities at that point. Rodents in RW on the other hand were found to be infested with lower numbers of ticks and on fewer occasions, albeit two larvae were found on them as late as October.

Table 4.10. Monthly distribution of tick numbers infesting individual rodents in BW and RW in 2010. (n) = nymph.

Month	BW	RW
April	-	-
May	-	-
June	1, 2, 3, 5	2, 3
July	-	1 (n)
August	1	1, 1, 1
September	2, 3, 3	-
October	-	2

Ticks collected from rodents were identified using the morphological keys described in section 2.2.1. Apart from the *I. ricinus* specimen described above, no further tick species were observed in 2009. In 2010, a total of 11 rodents infested with *I. trianguliceps* ticks was found. They consisted of two wood mice (infested with one and five larvae, respectively) and one bank vole infested with one larva from BW, and three wood mice (infested with one nymph, one larva and three larvae, respectively) and five bank voles (with one larva each) from RW.

4.3 Discussion

4.3.1 Habitats, plants, soil types and host animals

The described habitat compositions demonstrate that BW is poorer in plant species than RW, most likely due to its soil with a higher moisture and lower pH, its increased exposure to wind by lying on the northeastern face of a hillside, and the domination of *T. baccata*, which limits the availability of light to other plant species. While BW differs from more

common types of woodland habitats found in SW England that are dominated by broad-leaved tree species, it also differs from the ecological conditions found in the ecotonal habitat of RW. Here, more plant species growing at higher densities could be found, which is mostly due to its more favourable conditions, including higher availability of light for plants in lower vegetation layers thanks to a lighter, deciduous canopy, and reduced exposure to wind. Both habitats supplied a deep, moist layer of vegetation on the ground that provided cover and sufficient moisture levels above 80% required to ensure tick survival (e.g. Milne 1950). In a study on the relationship between vegetation habitats and high incidences of *I. ricinus*-associated TBE in the Czech Republic, Daniel et al. (1998) ranked ecotonal zones as the areas with the highest predictive risks for TBE occurrence, while coniferous woodlands were considered to be virtually without TBE risk. Similar observations were made for LB in North America (Allan et al. 2003) and for louping-ill virus (LIV) in a theoretical model (Jones et al. 2010). The increased surface area of the habitat edges due to the dense network of footpaths and bordering grasslands and pastures further helps to increase the species richness in RW. The density of highly frequented footpaths may also increase the likelihood of interactions between questing ticks and humans, or, in the case of dog-walkers, with their animals, which can also be affected by borreliosis (May et al., 1990, cited in Ogden et al., 2000).

In a study from 1995, Kurtenbach et al. assessed different habitats in a nature reserve in western Germany. Two of the five biotopes described are to some extent comparable to BW and RW in terms of soil and vegetation types: Like BW, “Biotope 5” contained acidic and clayey soil with impeded drainage, a deep, moist leaf litter layer and occasionally sparse cover with ground vegetation, but *T. baccata* as dominating tree species was absent. “Biotope 2” resembles the denser and more diverse vegetation cover found in RW, with *Acer* spp. and F*raxinus* sp. as the main tree species, and *Hedera helix* and *Clematis vitalba* occurring in the undergrowth above a drier and more permeable soil (Kurtenbach et al. 1995).

Consistent with these findings are also the reduced densities of some animals (medium-sized mammals and birds) that could act as potential hosts for ticks in BW. Again, this is most likely due to the harsher ecological conditions in this habitat, and the overlapping biotope fringes in the ecotonal habitat of RW. The comparable occurrence of large mammal hosts, such as roe deer and sheep in and near both habitats, suggests that both habitats are

capable of sustaining permanent tick populations, independent of the presence of small rodents (Gray et al. 1992). No other deer species, such as fallow deer or Reeve's muntjac, were observed. Unlike deer, which have been shown to be incompetent hosts for borrelia spirochaetes (Telford et al. 1988, Jaenson & Talleklint 1992), sheep can maintain an enzootic cycle of borrelia through co-feeding of infected and uninfected ticks (Ogden et al. 1997).

In an analysis of the presence of host animal for *I. ricinus* in a *bocage* landscape in Central France, Vourc'h et al. (2008) showed different host compositions in woodlands, pastures and in hedgerows that separated the two other habitats as ecotones. They did not observe any marked differences in the presence of large and medium-sized mammals between the three habitats, but found the highest number of bird species in the hedgerow ecotone, followed by the woodland habitat, similar to our observations.

Apart from the occurrence of passerine birds and small rodents (mice and voles) as most important hosts for immature tick stages, which will be covered separately in sections 4.3.3 - 4.3.5, other relevant host species observed included grey squirrels, and, less frequently, rabbits, hedgehogs and pheasants. Badger and foxes were recorded in both habitats, but have been reported not to feed many *I. ricinus* ticks (Milne 1949; Couper et al. 2010). Medium-sized mammals may also not act as equally strong reproductive hosts for ticks as deer, but can support an enzootic cycle of borrelia as reservoir hosts (eg Gray et al. 1994, 1998; Craine et al. 1995, 1997, Kurtenbach et al. 1998) During the summer, squirrels tend to feed predominantly in the tree canopy rather than on the ground (Kenward & Tonkin 1986, Kenward et al. 1998), thereby limiting their role as hosts when questing ticks would still be active (Gray 1991). Grey squirrels are generally less arboreal than red squirrels, but have been described to be more so in coniferous (such as BW) than in deciduous woodlands (Smith, 1999, cited in: Gurnell & Hare 2008). They are, on the other hand, known to be more diurnally active (Kenward & Tonkin 1986), which would bring them into more frequent contact with the likewise diurnal *I. ricinus* (Lees & Milne 1951, see 4.3.2). Mice and voles, generally considered major hosts for ticks, are more nocturnal and biphasically active (Wolton 1983, Montgomery & Gurnell 1985, cited in Craine et al. 1995), thereby reducing their frequencies of tick contacts. Similarly, hedgehogs can be heavily infested with ticks (mainly *I. ricinus* and *I. hexagonus*, see Hillyard [1996], Churchfield [2008]), but are almost entirely absent from coniferous woodland (Churchfield

2008). Due to their nocturnal activity their presence was difficult to assess. The role of other insectivores, such as shrews, as potential reservoir hosts for borrelia has been described by Talleklint & Jaenson (1993, 1994), but due to their legal protection these animals could not be captured during this study.

The importance of pheasants both as reservoirs for borrelia and for *I. ricinus* in southern England has been described previously (Craine et al. 1995, 1997; Kurtenbach et al. 1998a; Kurtenbach et al. 1998b). They mainly feed nymphal ticks (Kurtenbach et al. 1998a) and act as an avian reservoir for *B. garinii* and *B. valaisiana* (Kurtenbach et al. 1998b).

4.3.2 Collected ticks

High variations in tick numbers as shown in this study have been previously reported from other European locations (such as Germany, Kurtenbach et al. 1995; Sweden, Talleklint & Jaenson 1996; Ireland, Gray et al. 1999; or The Netherlands, Gassner et al. 2008, 2010). These fluctuations in the tick populations have been described to be the product of seasonal and annual variations in tick recruitment (by feeding on a host) and loss by starvation (Randolph & Steele 1985). They demonstrate the interaction of two challenges that ticks have to face: on the one hand they need to quest in the vegetation for vertebrate hosts, but are very susceptible to desiccation during their vertical movements (Lees 1946; Knülle & Rudolph 1982, cited in Randolph & Storey 1999) and are generally inactive when moisture levels remain below 80%-85% RH for a longer time (Daniel et al. 1998; Macleod 1938, Lees 1946, Kahl & Knülle 1988, cited in Gray 1991). They therefore need to return to the moist ground layers below the vegetation (Lees & Milne 1951) to restore their water levels through active and passive uptake (Knülle & Rudolph 1982, Needham & Teel 1991, Sonenshine 1991, cited in Randolph & Storey 1999), normally following a diurnal pattern (Belozerov 1982, cited in Randolph & Storey 1999). Therefore, variations in seasonal activity as observed in this study can be explained by small-scale responses of ticks to temperature and humidity (van Es 1999; Jensen 2000; Perret 2000, cited in Gray 2002).

The higher densities in BW than in RW for overall tick numbers, nymphs and adults confirm findings by Lindstrom & Jaenson (2003) about higher density in woodlands than in open habitats, such as ecotones. Another explanation for the discrepancies in tick numbers has been put forward by Craine et al. (1995), in that denser ground cover by low vegetation (as seen in RW) may prevent ticks from being sampled by blanket-dragging,

while they would be more accessible in the reduced vegetation cover in BW (see also Tack et al. 2011). While relative humidities in the two habitats were measured below the 80% threshold mentioned above (see Figs. 4.1, 4.2), it appears very likely that these reflect only short-term observations and not longer trends, as questing ticks could still be collected from the vegetation on these occasions. The seasonal variations that could be observed in 2010 differed between the two habitats. The peak of tick activity in BW took place around May, followed by a gradual decline over summer and autumn. This confirms previous findings from Wales, Southern England and Ireland (Steele & Randolph 1985; Randolph & Storey 1999; Randolph et al. 1999; reviewed in Kurtenbach et al. 2006). Albeit on a smaller scale, questing nymphs and adults in RW were found to follow a more bimodal pattern, as commonly seen in Central Europe, northern England and Scotland (reviewed in Gray 1991; Pietzsch et al. 2005; Kurtenbach et al. 2006). It should be noted, however, that the number of ticks sampled in 2006-2009 was very low, which could limit the confidence in the conclusions drawn from these data.

4.3.3 Ticks collected from birds

The importance of passerine birds as major hosts for both *I. ricinus* ticks and for certain borrelia genospecies is well established (eg Humair et al. 1993; Olsen et al. 1995b; Hanincova et al. 2003; Comstedt et al. 2006; Taragel'ova et al. 2008; Dubska et al. 2009). Similar to our findings, these studies also describe higher densities of nymphal and adult ticks infesting songbirds than larvae, and higher nymphal infestation rates than on rodents. Our findings also partially confirm the role of some songbird species, such as great tits, as a common host, while other species, such as blackbirds and robins, were less heavily infested than previously described (Humair et al. 1993; Hanincova et al. 2003, Taragel'ova et al. 2008). Greenfinches were the most heavily infested birds in our study, but featured far less prominently in the publications mentioned above. Interestingly, passerine birds do not show any immunity against ticks infestations, unlike some rodents (Dizij & Kurtenbach 1995; see 4.3.5), which might indicate their longer co-evolutionary adaptations (Heylen et al. 2010).

Due to their migratory patterns, passerine birds have a great influence on the distribution of both *Ixodes* ticks and of borrelia spirochaetes, as shown by the transhemispheric cycle between marine birds, *I. uriae* and *B. garinii* (Olsen et al. 1993, 1995a; Larsson et al. 2007); the possibility of migrating birds establishing new populations

of ticks will be discussed later.

4.3.4 Trapped rodents

Although fewer rodents were analysed in this study than in previous works, some of the findings allow comparison to earlier results. Gassner (2010) found densities for wood mice similar to ours at different sites in the Netherlands, albeit at habitats with sandy soils and different ecologies. De Boer et al. (1993) helped to establish the role of wood mice as reservoir hosts for *B. burgdorferi* s.l. spirochaetes in Europe. In their study, they trapped rodents in the Netherlands in short trapping sessions of three consecutive nights between May and December 1990. They found higher numbers of wood mice, but no bank voles in two of three different sites. They did not give an estimate of the area sizes that were sampled or of rodent densities, thereby limiting their comparability. Kurtenbach et al. (1995) compared the abundance of rodents in different biotopes (or habitats comparable to BW and RW, see 4.3.1) and found strong variations between different habitats and between different years. There, mean densities of wood mice ranged from 0.6 to 9.5 animals per hectare for a habitat analog to RW (calculated densities per 100 m^2 for 2009 and 2010: 1.18 and 1.25), and between 2.6 and 11.2 for a habitat resembling BW (calculated densities per 100 m^2 for 2009 and 2010: 2.17 and 0.92). The results in this study were obtained from a much smaller scale, and the extrapolation from a density per 100 m^2 to one per hectare (= 10.000 m^2) would clearly inflate any errors.

While it has previously been described that rodents in Britain become relatively scarce in May-July (Alibhai & Gipps 1985, Flowerdew 1985, Mallorie & Flowerdew 1994, cited in Craine et al. 1995), these results are not supported by my findings, which showed rodent densities reaching their peaks between June and September in 2010, and in August 2009. Kurtenbach et al. (1995) described a unimodal pattern with peaks in July for a German habitat analog to BW, and in September/October for the habitat resembling RW. Kikkawa (1964) tested rodent abundances in a woodland in southern England that resembled the wooded patches in RW and found unimodal patterns with a steep increase in population sizes from June onwards, leading to peaks in autumn, but no absence of rodents in May-July as described above. He did, however, show that certain parts of rodent populations remain less trappable than others. The highest proportion of bank voles to avoid traps was described for the autumn months, while wood mice became least trappable during summer, which was also reflected in the high numbers of recaptured animals in our study.

In a comparison of different habitats in France, Boyard et al. (2008) found higher numbers of rodents in a hedgerow ecotone than in woodlands, which supports the results for RW and BW, respectively. They also found comparable densities (between 2.57 and 4.06 animals per 100 trap nights compared to 3.00-4.73 in Bath in 2010) for wood mice and bank voles, although bank voles were more common than wood mice.

LoGiudice et al. (2003) described the consequences of reduced species diversity in fragmented habitats compared to intact woodlands in North America (while the opposite was observed in RW vs. BW). They found similar densities of white-footed mice (*Peromyscus leucopus*) in both habitats, but the reduced number of other host species that could act as dilution hosts in the fragmented sites led to an increased prevalence of borrelia spirochaetes in mice. It could also suggest that the higher diversity observed in the ecotonal habitat of RW could be the result of older fragmentations and a newly-established balance. On the other hand, a comparison of habitat types in California, USA, found higher numbers of rodents in ecotones than in woodlands (Eisen et al. 2004). These findings also demonstrate the variation in habitat types and the differences in the ecology of Lyme borreliosis and its vectors, as reviewed by Kurtenbach et al. (2006).

4.3.5 Ticks infesting rodents

Fewer ticks were found on rodents in RW than in BW in 2009, while this reversed in 2010. The late onset of the trapping period in July 2009 might be one explanation for the absence of ticks on rodents, as high densities of wood mice in RW were recorded for June 2010. Craine et al. (1995) indicated that rodents foraging in the litter layer might be better indicators for the presence of larvae than dragging, which is normally limited to suitable weather conditions (a wet blanket after rain commonly fails to collect any ticks at all), although larvae were not quantified in this study.

The infestation rate of larvae on rodents found in 2010 in both habitats were much lower than those recorded in previous studies in Europe. Kurtenbach et al. (1995) found mean numbers of larvae in a habitat analog to RW to range from 14.0 to 24.4 larvae per wood mouse and from 11.2 to 14.4 larvae per bank vole, while the habitat equivalent to BW showed mean numbers between 68.2 and 73.0 larvae per wood mouse and between 32.2 and 37.0 larvae per bank vole. These figures are by the factor 100 higher than those recorded in this study in 2010 (0.40 for BW and 0.42 for RW, respectively), and although partially a different technique was employed by Kurtenbach et al. (replete ticks were

allowed to drop off from captured rodents over a water basin instead of searching the captured rodent directly in the field), it appears highly unlikely that many feeding larvae would have been consistently overlooked. Gray et al. (1999) found 49.0% of all wood mice and 35.1% of bank voles in a study in Ireland to be infested with larvae, compared with 19.1%-25.8% for wood mice and 11.8% for bank voles in Bath in 2010. The intensity of larval infestations per rodent that Gray et al. described, however, were much closer to those observed in this study. They found 2.12 larvae per wood mouse and 0.95 larvae per bank vole, which is comparable to the intensities shown in Table 4.8. In the Netherlands, De Boer et al. (1993) described much lower mean infestation rates between 0.70 and 4.03 larvae per wood mouse, which are more comparable to those observed in Bath in 2010, but were only measured over a shorter period of time. These variations are most likely due to different experimental designs as well as high variations in tick and rodent abundances, which in turn are determined by several biotic and abiotic factors.

The observation made in this study that more larvae infested wood mice than bank voles supports most previous findings (eg De Boer et al. 1993; Kurtenbach et al. 1995; Gray et al. 1999). This phenomenon is due to an acquired immune resistance of bank voles against infestations with *I. ricinus* (Dizij & Kurtenbach 1995). It has been shown that high levels of testosterone could reverse this resistance (Hughes & Randolph 2001a, b), which suggested sexually mature male rodents to be most susceptible hosts for ticks, although this could not be confirmed by this study.

Previous authors have compared the ratio of larvae (L) to nymphs (N) feeding on wood mice (Gray 2002) and on bank voles (Randolph & Storey 1999). Gray (2002) found a much higher L:N ratio in Ireland of 649:1 (Gray et al. 1999) than in studies from continental Europe, such as 5:1 (Netherlands, in: de Boer et al. 1993), 20:1 (Switzerland, Humair et al. 1999) or 147:1 (Germany, Kurtenbach et al. 1995). The results from this study indicate a ratio of 29:1 for 2010, which would make them comparable to those studies from Europe, while the mean number of larvae per mouse would be closer to those described by Gray and colleagues for Ireland (see above). The absence of nymphs on rodent hosts has implications for the ecology of borrelia spirochaetes and the frequency of different, host-specific genotypes. Not only are nymphs far more efficient in transmitting borrelia than larvae (Ogden et al. 1998), but if nymphal ticks feed preferentially on birds than on rodents, this would shift the frequency of borrelia genospecies away from rodent-

associated *B. afzelii*, and towards bird-borne genospecies, such as *B. garinii* and *B. valaisiana* (reviewed by Kurtenbach et al. 2002). Indeed, Vollmer et al. (2011) could show that *B. afzelii* was entirely absent from questing ticks sampled in BW between 2006 and 2008, but present in ticks from RW from 2008. One possible explanation for this observation would be the introduction of *B. afzelii* through infected host animals in recent and independent events. This hypothesis is supported by the phylogenetic analysis of *B. afzelii* samples conducted by Vollmer et al. (2011).

4.4 Conclusions

In summary, this chapter analysed the differences in habitat ecology, host diversity, tick abundance, borrelia infection rates and prevalence of tick-infested rodents in two different habitats near Bath, UK. It summarises for the first time biotic and abiotic conditions that help to facilitate the maintenance of enzootic cycles of *B. burgdorferi* s.l. in a woodland and in an ecotonal habitat. While the woodland site contained higher numbers of questing ticks in 2010, the ecotone between woodland and open areas harboured a higher diversity of potential tick host species, especially more songbirds, and showed slightly higher densities of small rodents. Both habitats showed extremely low numbers of immature ticks infesting rodents, which might indicate that rodents have been replace by other hosts due to either genetic and/or ecological factors that will be analysed in other parts of this work.

5. Molecular identification of host blood meal source in *I. ricinus*

5.1 Introduction

The need to analyse interactions of ectoparasites and hosts is fundamental to an understanding of the ecology of vector-borne infectious diseases such as human Lyme Borreliosis (LB). *I. ricinus* has been identified as the main vector in Western and Central Europe (e.g. Gray, 1991), and the identification of reservoir host species would allow specific management measures to control enzootic cycles of the disease. The reservoir potential of hosts for *Borrelia burgdorferi* s.l. in Europe has been assessed (Gern et al., 1998; Gern & Humair, 2002), but these studies do not claim to be complete and fully accurate. A definitive, but lengthy and labour-intensive procedure to establish reservoir identification includes the trapping of animals, temporary captivity to allow engorged ticks to detach from the animals, and subsequent tick xenodiagnosis (the exposure of a parasite-free host to the tick, followed by detection of the pathogen in the host). As described by Humair et al. (2007), this technique might lead to a biased interpretation in the role of small animals such as rodents, due to the relative ease with which these animals can be trapped and maintained, as compared to birds or larger mammals. It is also impossible to collect engorged ticks from the vegetation by blanket dragging, since only actively questing ticks are captured by this method (Sonenshine 1991, cited in Kirstein & Gray, 1996).

Molecular methods to study the blood meals of ectoparasites have by now been established for several groups of ectoparasites as vectors of human diseases. These include *Culex* spp. (e.g. Apperson et al., 2004) and *Anopheles* spp. mosquitoes (Scott et al., 2006; Kent et al., 2007) as vectors of West Nile Virus (WNV) and human malaria, respectively, black flies (*Simulium* spp.) transmitting *Onchocerca volvulus* nematodes (Basáñez et al., 2007), sand flies (Phlebotominae) as vectors of *Leishmania* spp. (Haouas et al., 2007), and tsetse flies (*Glossina* spp.) that transmit African trypanosomes (Steuber et al., 2005). Blood meals in ticks have also been examined, both in preserved specimens (Tobolewski et al., 1992) and in field-collected *I. ricinus* ticks in several studies in Ireland, Spain, Switzerland and Germany (Kirstein & Gray, 1996; Pichon et al., 2003, 2005, 2006; Estrada-Peña et al., 2005; Humair et al., 2007; Moran Cadenas et al., 2007).

The main challenges to identify host DNA in ticks are the small amounts of blood meal

leftovers in the midgut of a tick and the long time that has passed between the uptake of a blood meal and the analysis of the subsequent tick stage that has been collected while questing. Therefore, in all studies mentioned above that have attempted to analyse tick blood meals PCR techniques were employed to amplify remnants of host DNA in the ticks. Several candidate genes have been selected, of which small fragments were amplified in order to discriminate between different host groups, genera or even species. Kirstein and Gray (1996) used a fragment of the mitochondrial housekeeping gene *cytochrome b* (*cytb*), while later studies by Pichon et al. (2003; 2005) used the nuclear ribosomal 18S gene, or a combination of 18S and mitochondrial 12S rDNA (Pichon et al., 2006) as markers. Further studies by Humair et al. (2007) and Moran Cadenas et al. (2007) focused on different fragments of 12S rDNA by designing novel primers and managed to achieve similar sensitivity, but higher specificity than the earlier studies by Pichon et al. (2003; 2005). While the sequencing of obtained PCR products would deliver direct and straightforward results, the cost of high-throughput analysis of a large number of samples would be a limiting factor. Reverse-line blot hybridisation (RLB) offers a more cost-effective approach for large scale assays (for a detailed comparison of various BMA techniques see Kent, 2009). RLB involves the binding of amino-bound host-specific oligonucleotide probes to an activated membrane. Biotin-labelled PCR products hybridise with suitable oligo probes, which is visualised by incubation with streptavidin peroxidase and a chemiluminescent substrate solution (see section 2.2.6 for a detailed protocol). The use of a standard 45-lane mini blotter system allows the testing of 44 samples (including positive and negative controls) and one marker lane against 44 different oligo probes, and oligo-coupled membranes can be prepared in advance, thereby allowing a faster processing of large numbers of samples. Other approaches have been tested as a proof of principle, such as 2D-electrophoresis (Vennestrom & Jensen, 2007) or mass spectrometry (Wickramasekara et al., 2008), which would enable to detect host DNA in ticks up to 11 months after their last blood meal, but are currently less well established.

The studies mentioned above have assessed blood-feeding behaviour of *I. ricinus* ticks in Ireland (Kirstein & Gray, 1996; Pichon et al., 2003; 2005) as well as in Northwest Spain (Estrada-Peña et al., 2005), Switzerland (Humair et al., 2007; Moran Cadenas et al., 2007) and Germany (Pichon et al., 2006), but so far no molecular analysis of tick hosts from Southwest England has been conducted. The relative scarcity of rodent-associated *B.*

afzelii in habitats near Bath, UK, has been described by Vollmer et al. (2011), while it has also been observed that ticks in this area exhibit unique patterns of activity. Unlike the bimodal pattern found for questing ticks in Northern England, Ireland, Scotland and Europe, with activity peaks in spring and in autumn, a more unimodal pattern with a peak in late spring for all stages has been described in Southern England (Gray 1991; Craine et al., 1995; reviewed in Kurtenbach et al., 2006). These findings highlight the need to integrate ecological findings of tick and host animal phenology, as described in chapter 4 of this thesis, with a molecular analysis of the feeding behaviour of ticks from the same habitats. It should also be assessed whether and how the interactions of questing and feeding ticks, the availability of host animals, and LB spirochaetes selected for by the vertebrate hosts and the ticks behaviour affect the ecology of Lyme Borreliosis in a specific habitat.

Apart from the small-scale analysis of samples from Britain, this study also attempts to examine on a larger geographical scale differences in the phenology of *I. ricinus* populations that manifest themselves on a molecular level. A recent study by Dinnis et al. (2011, in preparation) as well as chapter 3 of this study could show that tick populations sampled in Britain and in Latvia exhibited significant phylogeographic structuring, as well as huge differences in their prevalence for different borrelia genospecies (Etti et al., 2003; Vollmer et al., 2011). It is therefore inviting to compare the same tick samples for differences in their respective blood meal host compositions, especially with regard to their infection with borrelia spirochaetes. However, a definitive analysis of their host-seeking patterns would require a fully comprehensive census of host animals that were available in each habitat at the specific point of questing for each tick, which would be beyond the scope of this study.

Therefore, the aim of this chapter was to test the following hypotheses:

- Ticks collected in two different habitats in Southern England exhibit differences in their utilisation of vertebrate hosts as shown by their blood meal analysis (BMA).
- Infection with borrelia spirochaetes alters the feeding patterns of the ticks.
- *I. ricinus* ticks from Southern England and from Latvia are not only phylogenetically distinct, but also show differences in their utilisation of host species, which might affect their vector competence for borrelia spirochaetes.

5.2 Results

5.2.1 Comparison of two habitats near Bath, UK

5.2.1.1 Overall comparison between two sites: Bathampton Woods and Rainbow Woods

A total number of 249 questing *I. ricinus* ticks that had been collected by blanket dragging between 2006 and 2008 was included in this section. Of these, 126 nymphs and 17 adults had been collected in Bathampton Woods (BW), while 62 nymphs and 44 adults were from Rainbow Woods (RW). In a previous study, these ticks had been tested for the presence of *Borrelia burgdorferi* s.l. spirochetes (data included in Vollmer et al., 2011). Table 5.1 summarizes the collection data.

Table 5.1. Overview of borrelia-positive and -negative *I. ricinus* ticks collected from BW and RW between 2006 and 2008 used in this study.

		borrelia-positive	borrelia-negative	Total
BW	nymphs	36	90	**126**
	adults	1	16	**17**
RW	nymphs	33	29	**62**
	adults	2	42	**44**
Total		**72**	**177**	**249**

The ticks were analysed for vertebrate host DNA they had taken up during previous blood meals by applying a reverse line blot (RLB) assay developed by Rijpkema et al. (1995) and modified by Humair et al. (2007), which is described in detail in section 2.2.6. As an example, Fig. 5.1 shows a scanned image of a developed film slide that depicts the results from 32 ticks (28 nymphs, four females) collected in BW in 2007.

Figure 5.2 shows the distribution of positive signals in all ticks (nymphs and adults) from each site, while Fig. 5.3 depicts the positive signals distinguished between nymphal and adult ticks. Ticks from the two habitats are compared in summary in Table 5.2. Overall, both nymphal and adults from RW showed a higher diversity of blood-meal hosts than ticks from BW. DNA from the following host species or groups was found in ticks from RW that were absent in ticks from BW: fallow deer (*Dama dama*)/sika deer (*Cervus nippon*), sheep (*Ovis aries*), European fox (*Vulpes vulpes*), weasel (*Mustela nivalis*), water shrew (*Neomys fodiens*), European rabbit (*Oryctolagus cuniculus*), field vole (*Microtus*

agrestis)/harvest mouse (*Micromys minutus*); whereas tits (*Parus* spp.) were found exclusively in BW. No signals at all were found for bank voles (*Myodes glareolus*), squirrels (*Sciurus* spp.), brown hares (*Lepus europaeus*), hedgehogs (*Erinaceus europaeus*), or for common pheasants (*Phasianus colchicus*).

A significantly ($P < 0.05$; all significance tests in this chapter: Fisher's exact probability test) higher proportion (97.0%) of ticks from BW than ticks from RW (84.2%) was found to be positive for the mammalian oligo probe, and over 40% from both sites hybridised with the artiodactyl probe (Artiodactyla are even-toed ungulates, a group of mammals that includes cattle, sheep and deer). Significantly more nymphal and overall ticks from RW than from BW had previously fed on birds, while the adults ticks did not show such a difference. No differences between the two sites were observed in the proportion of ticks that had fed on small rodents, such as wood mice (*Apodemus sylvaticus*) or yellow-necked mice (*A. flavicollis*).

Ticks from RW showed a significantly higher number of mixed signals for the combinations of artiodactyls + rodents, artiodactyls + birds, and for rodents + birds than ticks from BW, while about 20% of the samples from both sides were double positive for the mammal and bird probes, and between 7% and 10% were double positive for signals from badgers and artiodactyls.

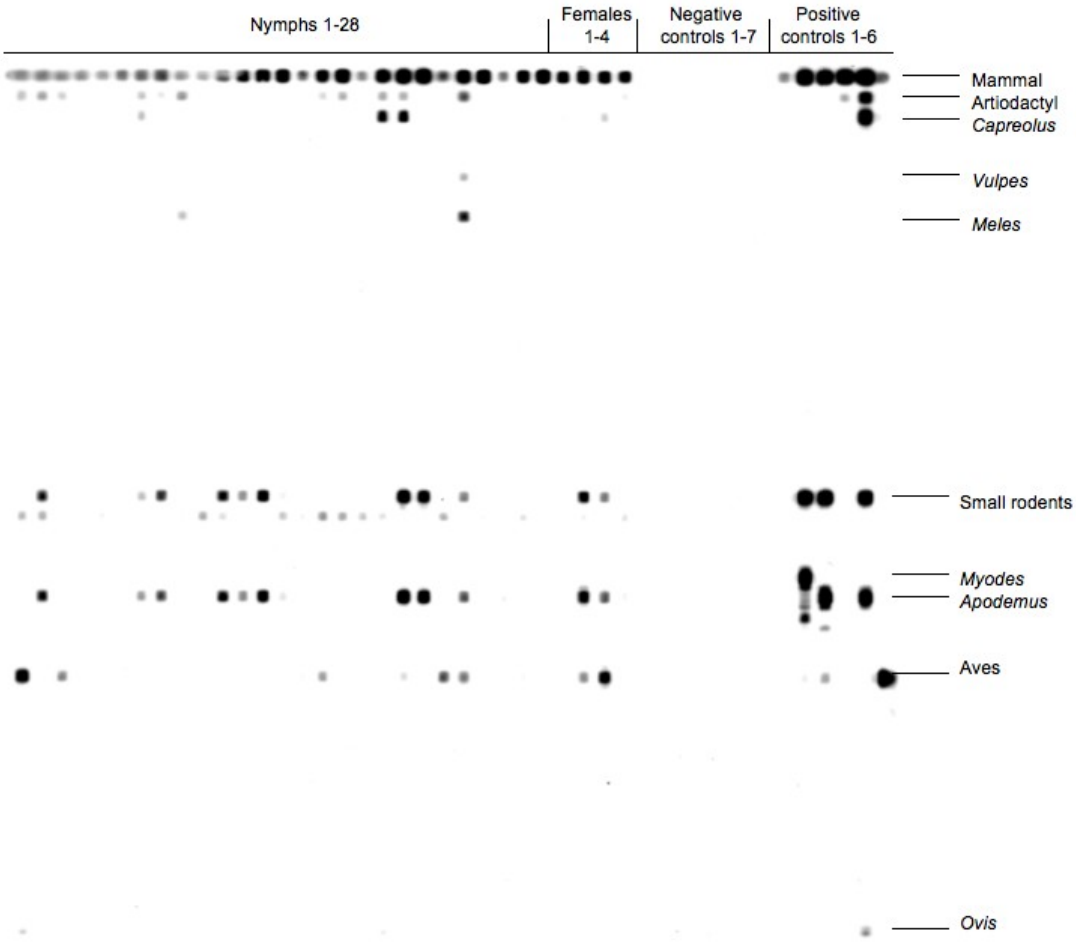


Fig. 5.1. Reverse line blot assay for the identification of vertebrate host groups/species in questing *I. ricinus* ticks collected in BW in 2007. Positive controls used were: 1) *Sciurus carolinensis*; 2) *Myodes glareolus*; 3) *Apodemus sylvaticus*; 4) *Ovis aries*; 5) *Capreolus capreolus*; 6) *Gallus gallus*.

Table 5.2. Proportions of nymphal, adult and overall ticks from BW and RW showing positive signals for one or more of 20 RLB oligo probes. The most important host groups are shown in bold and groups differing significantly ($P < 0.05$, Fisher's exact test) from each other are denoted by asterisks.

Host DNA identification	Nymphs		Adults		Total	
	BW	RW	BW	RW	BW	RW
Mammalia	**113/117 (96.6%)**	**49/56 (87.5%)**	**16/16 (100%)**	**31/39 (79.5%)**	**129/133 (97.0%)***	**80/95 (84.2%)***
Artiodactyla	**55/117 (47.0%)**	**28/56 (50.0%)**	**3/16 (18.8%)**	**12/39 (30.8%)**	**58/133 (43.6%)**	**40/95 (42.1%)**
Capreolus	25/117 (21.4%)	17/56 (30.4%)	3/16 (18.8%)	8/39 (20.5%)	28/133 (21.1%)	25/95 (26.3%)
Dama/ C. nippon	-	6/56 (10.7%)	-	-	-	6/95 (6.3%)
Ovis	-	1/56 (1.8%)	-	2/39 (5.1%)	-	3/95 (3.2%)
Vulpes	-	6/56 (10.7%)	-	-	-	6/95 (6.3%)
Meles	14/117 (12.0%)	5/56 (8.9%)	2/16 (12.5%)	3/39 (7.7%)	16/133 (12.0%)	8/95 (8.4%)
Mustela	-	2/56 (3.6%)	-	-	-	2/95 (2.1%)
Sorex	-	-	-	-	-	-
Neomys	-	3/56 (5.4%)	-	-	-	3/95 (3.2%)
Oryctolagus	-	1/56 (1.8%)	-	1/39 (2.6%)	-	2/95 (2.1%)
Sciurus	-	-	-	-	-	-
Small rodents	**42/117 (35.9%)**	**18/56 (32.1%)**	**5/16 (31.3%)**	**15/39 (38.5%)**	**47/133 (35.3%)**	**33/95 (34.7%)**
Myodes	-	-	-	-	-	-
Apodemus	42/117 (35.9%)	18/56 (32.1%)	5/16 (31.3%)	14/39 (35.9%)	47/133 (35.3%)	32/95 (33.7%)
Micromys/ Microtus	-	2/56 (3.6%)	-	1/39 (2.6%)	-	3/95 (3.2%)
Aves	**31/117 (26.5%)***	**23/56 (41.1%)***	**2/16 (12.5%)**	**14/39 (35.9%)**	**33/133 (24.8%)***	**37/95 (39.0%)***
Turdus	4/117 (3.4%)	4/56 (7.1%)	1/16 (6.3%)	-	5/133 (3.8%)	4/95 (4.2%)
Erithacus	1/117 (0.9%)	1/56 (1.8%)	-	-	1/133 (0.8%)	1/95 (1.1%)
Parus	1/117 (0.9%)	-	-	-	1/133 (0.8%)	-
Mammal+ Aves	27/126 (21.4%)	16/62 (25.8%)	2/17 (11.8%)	6/44 (13.6%)	29/143 (20.3%)	22/106 (20.8%)
Artio+ Meles	13/117 (11.1%)	4/56 (7.1%)	1/16 (6.3%)	3/39 (7.7%)	14/133 (10.5%)	7/95 (7.4%)
Artio+ Rodent	1/117 (0.9%)*	14/56 (25.0%)*	1/16 (6.3%)	2/39 (5.1%)	2/133 (1.5%)*	16/95 (16.8%)*
Artio+ Aves	1/117 (0.9%)*	9/56 (16.1%)*	1/16 (6.3%)	3/39 (7.7%)	2/133 (1.5%)*	12/95 (12.6%)*
Rodent+ Aves	1/117 (0.9%)*	8/56 (14.3%)*	1/16 (6.3%)	2/39 (5.1%)	2/133 (1.5%)*	10/95 (10.5%)*
Total positives	**117/126 (92.9%)**	**56/62 (90.3%)**	**16/17 (94.1%)**	**39/44 (88.6%)**	**133/143 (93.0%)**	**95/106 (89.6%)**

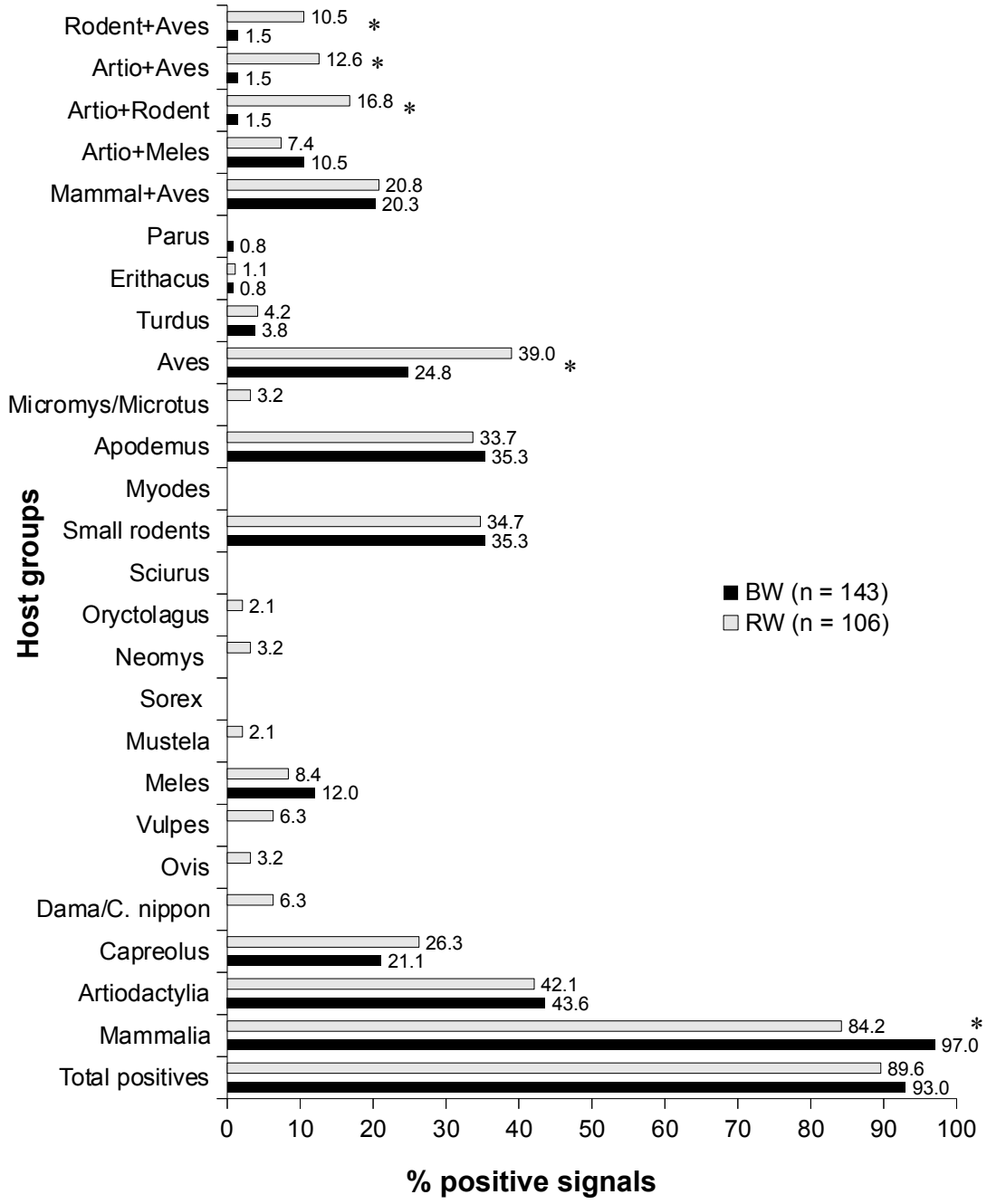


Fig. 5.2. Bar chart of percentages of positive signals found in all ticks (nymphs and adults) from BW and RW for 20 RLB oligo probes and combinations of double positive signals. Values that are significantly different ($P < 0.05$) between BW and RW are denoted by asterisks.

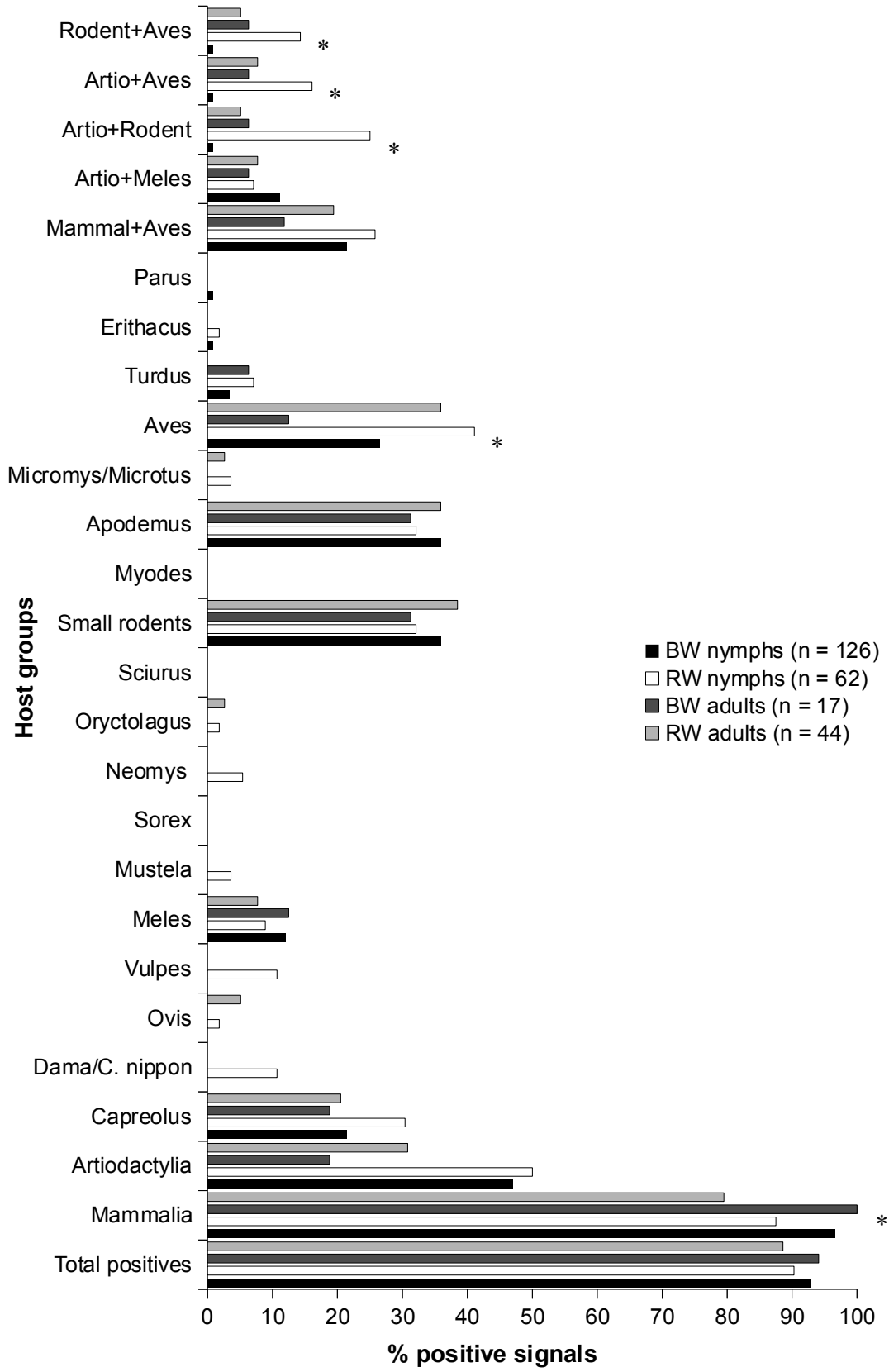


Fig. 5.3. Bar chart of percentages of positive signals found in nymphal and adult ticks from BW and RW for 20 RLB oligo probes and combinations of double positive signals. Values that are significantly different ($P < 0.05$) between BW and RW are denoted by asterisks.

5.2.1.2 Comparison of borrelia-negative and borrelia-positive ticks

When the two sites were distinguished in terms of the numbers of borrelia-positive and borrelia-negative ticks, further differences became visible. Borrelia-positive ticks from BW showed significantly more positive signals for the probes from artiodactyls, roe deer (*C. capreolus*), small rodents (including *Apodemus* mice) and had significantly more double positive signals for artiodactyls and rodents. A significantly higher proportion of ticks from RW that were positive for borrelia gave an overall positive result (100%) and were also positive for the mammalian probe as well as artiodactyls and roe deer. They furthermore showed significantly more double positive signals for artiodactyls and rodents and for artiodactyls and birds. The findings are summarized in Table 5.3 and depicted in Fig. 5.4.

Further differentiation of the borrelia-positive ticks according the respective borrelia genospecies found within them revealed further differences between the two sites, as shown in Table 5.4. Only a small proportion of the borrelia-positive ticks used in this analysis were adults, and they did not contribute to significant differences between the groups. A large proportion (about 90%) of the samples from both sites reacted with the mammalian probe, followed by 62-68% binding to the probe for artiodactyls. Borrelia-positive ticks from RW reacted less frequently (45.7%) to the probe for small rodents than those from BW (62.2%). They did, however, show a significantly higher proportion (34.3%) of bird-positive signals than ticks from BW (13.5%), and also for other hosts (mostly badgers and foxes).

B. afzelii was found in 12 of 35 (34.3%) ticks from RW (Table 5.4B), but was entirely absent in all 37 ticks from BW (Table 5.4A). Eleven out of these 12 (91.7%) ticks showed a positive signal for the mammal probe, of which seven (58.3%) reacted to the artiodactyl and four (33.3%) to the probe for roe deer. Five samples (41.7%) were found to bind to the oligo probe for small rodents (all from *Apodemus* mice), whereas three samples (25.0%) hybridized with the probe for birds. Signals from other host species were found in seven samples (58.3%).

B. garinii was found in a significantly higher proportion (54.1%) of all borrelia-positive ticks from BW than in the borrelia-positive ticks from RW (25.7%). All of these ticks from RW were positive for the mammalian and for the artiodactyl probe, while 85% and 70% of the ticks from BW reacted with these two probes, respectively. Although *B. garinii* is considered bird-borne, only 10% and 44.4% of the ticks from BW and RW, respectively,

that were positive for this genospecies were found to be positive for the bird probe. The findings for *B. valaisiana*-positive ticks, another genospecies associated with birds, and for ticks diagnosed with a mix of borrelia species showed little variation between the two sites.

Table 5.3. Comparison of borrelia-negative and borrelia-positive ticks from BW and RW. The most important host groups are shown in bold. Groups differing significantly ($P < 0.05$, Fisher's exact test) between borrelia-negative and borrelia-positive samples within a site are denoted by asterisks.

Host DNA identification	**BW borrelia- (n = 106)**	**BW borrelia+ (n = 37)**	**RW borrelia- (n = 71)**	**RW borrelia+ (n = 35)**
Mammalia	**96/100 (96.0%)**	**33/33 (100%)**	**47/60 (78.3%)***	**33/35 (94.3%)***
Artiodactyla	**35/100 (35.0%)***	**23/33 (69.7%)***	**16/60 (26.7%)***	**24/35 (68.6%)***
Capreolus	6/100 (6.0%)*	22/33 66.7%)*	9/60 (15.0%)*	16/35 (45.7%)*
Dama/ *C. nippon*	-	-	-	6/35 (17.1%)
Ovis	-	-	3/60 (5.0%)	-
Vulpes	-	-	-	6/35 (17.1%)
Meles	11/100 (11.0%)	5/33 (15.2%)	4/60 (6.7%)	4/35 (11.4%)
Oryctolagus	-		1/60 (1.7%)	1/35 (2.9%)
Small rodents	**26/100 (26.0%)***	**21/33 (63.6%)***	**18/60 (30.0%)**	**16/35 (45.7%)**
Apodemus	26/100 (26.0%)*	21/33 (63.6%)*	17/60 (28.3%)	16/35 (45.7%)
Micromys/ *Microtus*	-	-	-	3/35 (8.6%)
Aves	**28/100 (28.0%)**	**5/33 (15.2%)**	**25/60 (41.7%)**	**12/35 (34.3%)**
Mammal+ Aves	24/106 (22.6%)	5/33 (15.2%)	12/71 (16.9%)	10/35 (28.6%)
Artio+ Meles	9/100 (9.0%)	5/33 (15.2%)	4/60 (6.7%)	3/35 (8.6%)
Artio+ Rodent	7/100 (7.0%)*	19/33 (57.6%)*	1/60 (1.7%)*	15/35 (42.9%)*
Artio+ Aves	6/100 (6.0%)	5/33 (15.2%)	4/60 (6.7%)*	8/35 (22.9%)*
Rodent+ Aves	4/100 (4.0%)	4/33 (12.1%)	6/60 (10.0%)	5/35 (14.3%)
Total positives	**100/106 (94.3%)**	**33/37 (89.2%)**	**60/71 (84.5%)***	**35/35 (100%)***

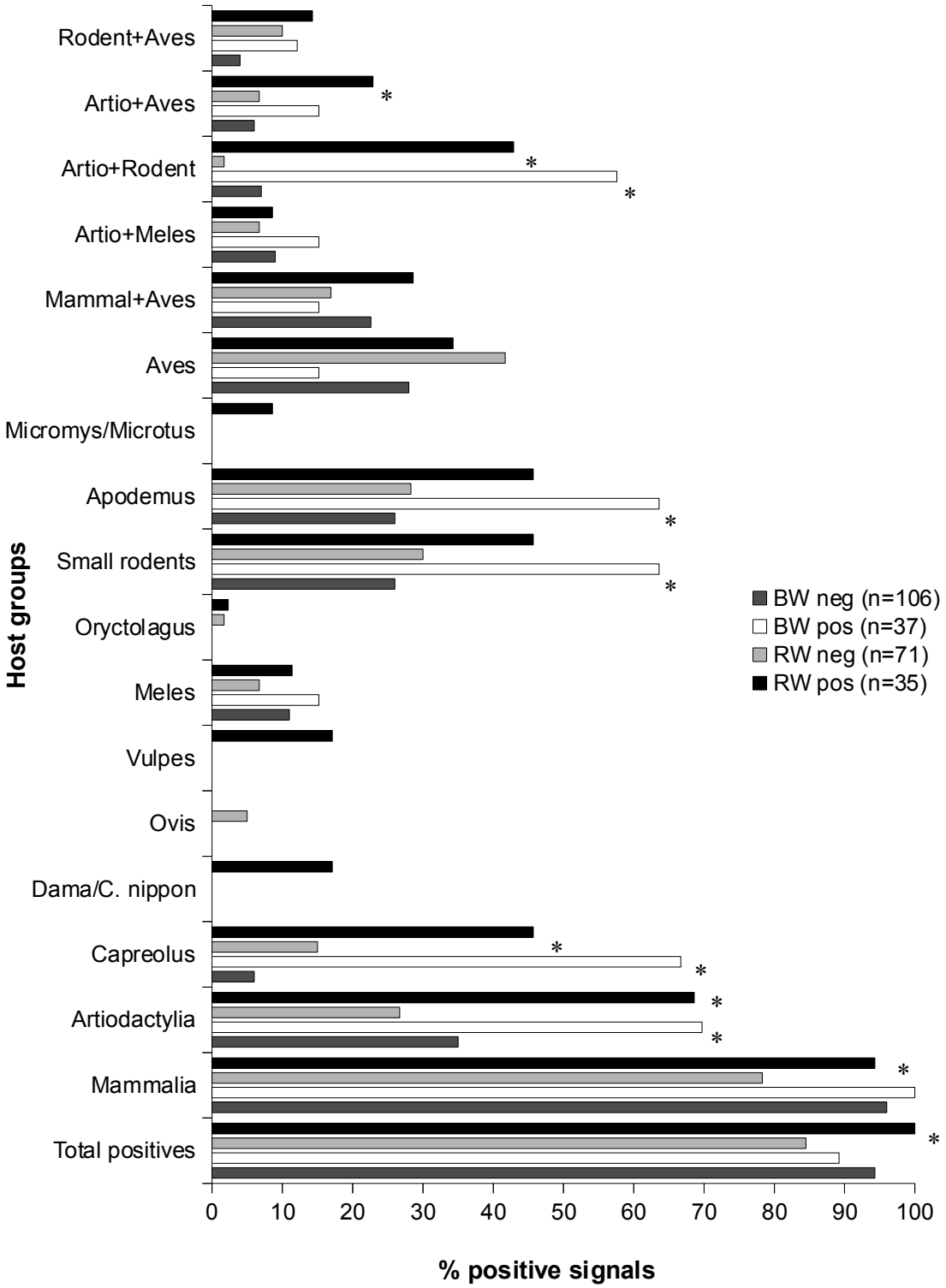


Fig. 5.4. Bar chart of percentages of positive RLB signals found in borrelia-positive and borrelia-negative ticks (nymphs and adults) from BW and RW for 12 RLB oligo probes and combinations of double positive signals. Values of groups differing significantly ($P < 0.05$, Fisher's exact test) between borrelia-negative and borrelia-positive samples within a site are denoted by asterisks.

Table 5.4. Distribution and frequency of positive host signals found in ticks tested positive for different borrelia genospecies, (A) from BW, and (B) from RW. Values that are significantly different ($P < 0.05$) between BW and RW are denoted by asterisks.

(A):

Host DNA identification	Ticks tested positive for borrelia genospecies									
	B. afzelii -		***B. garinii*** 20/37 (54.1%)*		***B. valaisiana*** 11/37 (29.7%)		**Mixed borrelia species** 6/37 (16.2%)		**Total (37)**	
	n	**a**	**n**	**a**	**n**	**a**	**n**	**a**	**n**	**a**
	-	**-**	20/37 (54.1%)*	**-**	**10/37 (27.0%)**	**1/37 (2.7%)**	**6/37 (16.2%)**	**-**	**36/37 (97.3%)**	**1/37 (2.7%)**
Mammal	-		17/20 (85.0%)		10/11 (90.9%)		6/6 (100%)		**33/37 (89.2%)**	
	-	-	17/20 (85.0%)	-	9/11 (81.8%)	1/11 (9.1%)	6/6 (100%)	-	**32/37 (86.5%)**	**1/37 (2.7%)**
Artiodactyls	-		14/20 (70.0%)		7/11 (63.6%)		2/6 (33.3%)		**23/37 (62.2%)**	
	-	-	14/20 (70.0%)	-	6/11 (54.5%)	1/11 (9.1%)	2/6 (33.3%)	-	**22/37 (59.5%)**	**1/37 (2.7%)**
Capreolus	-		13/20 (65.0%)		7/11 (63.6%)		2/6 (33.3%)		**22/37 (59.5%)**	
	-	-	13/20 (65.0%)	-	6/11 (54.5%)	1/11 (9.1%)	2/6 (33.3%)	-	**21/37 (56.8%)**	**1/37 (2.7%)**
Small rodents	-		14/20 (70.0%)		6/11 (54.5%)		3/6 (50.0%)		**23/37 (62.2%)**	
	-	-	14/20 (70.0%)	-	5/11 (45.5%)	1/11 (9.1%)	3/6 (50.0%)	-	**22/37 (59.5%)**	**1/37 (2.7%)**
Apodemus	-		14/20 (70.0%)		6/11 (54.5%)		3/6 (50.0%)		**23/37 (62.2%)**	
	-	-	14/20 (70.0%)	-	5/11 (45.5%)	1/11 (9.1%)	3/6 (50.0%)	-	**22/37 (59.5%)**	**1/37 (2.7%)**
Birds	-		2/20 (10.0%)		2/11 (18.2%)		1/6 (16.7%)		**5/37 (13.5%)***	
	-	-	2/20 (10.0%)	-	1/11 (9.1%)	1/11 (9.1%)	1/6 (16.7%)	-	**4/37 (10.8%)**	**1/37 (2.7%)**
Other hosts	-		2/20 (10.0%)*		2/11 (18.2%)		1/6 (16.7%)		**5/37 (13.5%)***	
	-	-	2/20 (10.0%)*	-	1/11 (9.1%)	1/11 (9.1%)	1/6 (16.7%)	-	**4/37 (10.8%)**	**1/37 (2.7%)**

Table 5.4. (continued)

(B):

Host DNA identification	Ticks tested positive for borrelia genospecies									
	B. afzelii 12/35 (34.3%)		***B. garinii*** 9/35 (25.7%)*		***B. valaisiana*** 11/35 (31.4%)		**Mixed borrelia species** 3/35 (8.6%)		**Total (35)**	
	n	**a**	**n**	**a**	**n**	**a**	**n**	**a**	**n**	**a**
	12/35 (34.3%)	-	9/35 (25.7%)*	-	**9/35 (25.7%)**	**2/35 (5.7%)**	3/35 (8.6%)	-	**33/35 (94.3%)**	**2/35 (5.7%)**
Mammal	11/12 (91.7%)		9/9 (100%)		10/11 (90.9%)		3/3 (100%)		**33/35 (94.3%)**	
	11/12 (91.7%)	-	9/9 (100%)	-	8/11 (72.7%)	2/11 (18.2%)	3/3 (100%)	-	**33/35 (94.3%)**	**2/35 (5.7%)**
Artiodactyls	7/12 (58.3%)		9/9 (100%)		7/11 (63.6%)		1/3 (33.3%)		**24/35 (68.6%)**	
	7/12 (58.3%)	-	9/9 (100%)	-	6/11 (54.5%)	1/11 (9.1%)	1/3 (33.3%)	-	**23/35 (65.7%)**	**1/35 (2.9%)**
Capreolus	4/12 (33.3%)		7/9 (77.8%)		4/11 (36.4%)		1/3 (33.3%)		**16/35 (45.7%)**	
	4/12 (33.3%)	-	7/9 (77.8%)	-	3/11 (27.3%)	1/11 (9.1%)	1/3 (33.3%)	-	**15/35 (42.9%)**	**1/35 (2.9%)**
Small rodents	5/12 (41.7%)		5/9 (55.6%)		5/11 (45.5%)		1/3 (33.3%)		**16/35 (45.7%)**	
	5/12 (41.7%)	-	5/9 (55.6%)	-	3/11 (27.3%)	2/11 (18.2%)	1/3 (33.3%)	-	**14/35 (40.0%)**	**2/35 (5.7%)**
Apodemus	5/12 (41.7%)		5/9 (55.6%)		5/11 (45.5%)		1/3 (33.3%)		**16/35 (45.7%)**	
	5/12 (41.7%)	-	5/9 (55.6%)	-	3/11 (27.3%)	2/11 (18.2%)	1/3 (33.3%)	-	**14/35 (40.0%)**	**2/35 (5.7%)**
Birds	3/12 (25.0%)		4/9 (44.4%)		4/11 (36.4%)		1/3 (33.3%)		**12/35 (34.3%)***	
	3/12 (25.0%)	-	4/9 (44.4%)	-	4/11 (36.4%)	-	1/3 (33.3%)	-	**12/35 (34.3%)***	-
Other hosts	7/12 (58.3%)		8/9 (88.9%)*		3/11 (27.3%)		2/3 (66.7%)		**20/35 (57.1%)***	
	7/12 (58.3%)	-	8/9 (88.9%)*	-	3/11 (27.3%)	-	2/3 (66.7%)	-	**20/35 (57.1%)***	-

5.2.2 Comparison between British and Latvian samples

5.2.2.1 Overall comparison between Britain and Latvia

In addition to the 249 British ticks from 2006-2008 analysed in the previous section, a further 215 ticks (69 nymphs and 146 adults) from Latvia were added to this study to allow comparison between two geographically separate populations. The Latvian ticks had been collected by collaborators between 2002 and 2006 in three areas around Riga and had also been included in a previous study on LB in Europe mentioned above (Vollmer et al., 2011). They consisted of 60 ticks from Babite, a sylvatic site, 62 from Jaunciemis (peridomestic, close to Riga), and 93 from Kemeri (mixed forest, peridomestic, bordering marshland). See Etti et al. (2003) for a more detailed description of the three sites.

The RLB results of the ticks from these two countries can be compared in Table 5.5. The percentages of positive signals from all ticks taken together are depicted in Fig. 5.5, which are further differentiated into nymphal and adult ticks in Fig. 5.6. Several host species were found exclusively in one of the two groups. British ticks were found to have been feeding on sheep (*Ovis aries*), badgers (*Meles meles*), weasels (*Mustela nivalis*), water shrews (*Neomys fodiens*), rabbits (*Oryctolagus cuniculus*), field voles/harvest mice (*Microtus arvalis*/*Micromys minutus*) and on one passerine bird, a member of the tit genus (*Parus* spp.). Latvian ticks on the other hand showed positive signals for wild boar (*Sus scrofa*), shrews (*Sorex* spp.), and European squirrels (*Sciurus vulgaris*). Ticks that had fed on red deer (*Cervus elaphus*) and bank voles (*Myodes glareolus*) were notably absent from both populations.

While ca. 90% of overall ticks and adults from both countries exhibited high percentages of ca. 90% of total positive signals, significantly more British (92.0%) than Latvian nymphs (76.8%) showed positive signals at all. About 85-90% of all ticks displayed positive signals for the mammalian probe, while significantly more British samples reacted to the artiodactyl (43.0%) and roe deer (*C. capreolus,* 23.2%) oligo probes, compared with 26.8% of the Latvian samples for artiodactyls and only 1.0% for roe deer, respectively. The rates of positive signals for small rodents and *Apodemus* spp. for both nymphs and adult ticks from Britain was tenfold higher (35.8-36.4%) than the respective rates found in Latvian ticks (3.5-3.8%). Adult British ticks as well as all British ticks taken together displayed a significantly higher prevalence of positive signals for the bird probe. Both stages also exhibited a significantly larger percentage of double positive signals from mammals and birds than Latvian ticks. Combinations of signals from artiodactyls and birds and from rodents and birds were also more frequently encountered in all British than in all Latvian ticks. A comparison of the Latvian ticks from the three different collection sites did not reveal any significant differences between host utilisation rates at different sites (data not shown).

Table 5.5. Proportions of nymphal, adult and overall ticks from Latvia and Britain showing positive signals for one or more of 20 RLB oligo probes. The most important host groups are shown in bold and groups differing significantly ($P < 0.05$, Fisher's exact test) from each other are denoted by asterisks.

Host DNA identification	Nymphs		Adults		Total	
	Latvia (n = 69)	Britain (n = 188)	Latvia (n = 146)	Britain (n = 61)	Latvia (n = 215)	Britain (n = 249)
Mammalia	**48/53 (90.6%)**	**162/173 (93.6%)**	**125/141 (88.7%)**	**47/55 (85.5%)**	**173/194 (89.2%)**	**209/228 (91.7%)**
Artiodactyla	**22/53 (41.5%)**	**83/173 (48.0%)**	**30/141 (21.2%)**	**15/55 (27.3%)**	**52/194 (26.8%)***	**98/228 (43.0%)***
Cervus	-	-	-	-	-	-
Capreolus	-	42/173 (24.3%)	2/141 (1.4%)*	11/55 (20.0%)*	2/194 (1.0%)*	53/228 (23.2%)*
Dama/ C. nippon	-	6/173 (3.5%)	1/141 (0.7%)	-	1/194 (0.5%)	6/228 (2.6%)
Ovis	-	1/173 (0.6%)	-	2/55 (3.6%)	-	3/228 (1.3%)
Sus	14/53 (26.4%)	-	14/141 (9.9%)	-	28/194 (14.4%)	-
Vulpes	-	6/173 (3.5%)	3/141 (2.1%)	-	3/194 (1.5%)	9/228 (3.9%)
Meles	-	19/173 (11.0%)	-	5/55 (9.1%)	-	26/228 (11.4%)
Mustela	-	2/173 (1.2%)	-	-	-	2/228 (0.8%)
Sorex	1/53 (1.9%)	-	1/141 (0.7%)		2/194 (1.0%)	-
Neomys	-	3/173 (1.7%)	-	-	-	3/228 (1.3%)
Oryctolagus	-	1/173 (0.6%)	-	1/55 (1.8%)	-	2/228 (0.8%)
Sciurus	-	-	2/141 (1.4%)	-	2/194 (1.0%)	-
Small rodents	**2/53 (3.8%)***	**62/173 (35.8%)***	**5/141 (3.5%)***	**20/55 (36.4%)***	**7/194 (3.6%)***	**82/228 (36.0%)***
Myodes	-	-	-	-	-	-
Apodemus	1/53 (1.9%)*	60/173 (34.7%)*	5/141 3.5%)*	19/55 (34.5%)*	7/194 (3.6%)*	82/228 (36.0%)*
Micromys/ Microtus	-	2/173 (1.2%)	-	1/55 (1.8%)	-	3/228 (1.3%)
Aves	**11/53 (20.8%)**	**54/173 (31.2%)**	**19/141 (13.5%)***	**16/55 (29.1%)***	**30/194 (15.5%)***	**70/228 (30.7%)***
Turdus	1/53 (1.9%)	8/173 (4.6%)	7/141 (5.0%)	1/55 (1.8%)	9/194 (4.6%)	9/228 (3.9%)
Erithacus	-	2/173 (1.2%)	-	-	1/194 (0.5%)	2/228 (0.8%)
Parus	-	1/173 (0.6%)	-	-	-	1/228 (0.4%)
Mammal+ Aves	**6/53 (11.3%)***	**43/173 (24.9%)***	**3/141 (2.1%)***	**8/55 (13.1%)***	**9/194 (4.6%)***	**51/228 (22.3%)***
Artio+ *Meles*	-	17/173 (9.8%)	-	4/55 (7.3%)	-	21/228 (9.2%)
Artio+ Rodent	-	39/173 (22.5%)	-	3/55 (5.5%)	-	42/228 (18.4%)
Artio+ Aves	3/53 (5.7%)	19/173 (11.0%)	2/141 (1.4%)	4/55 (7.3%)	5/194 (2.6%)*	23/228 (10.1%)*
Rodent+ Aves	1/53 (1.9%)	15/173 (8.7%)	-	3/55 (5.5%)	1/194 (0.5%)*	19/228 (8.3%)*
Total positives	**53/69 (76.8%)***	**173/188 (92.0%)***	**141/146 (96.6%)**	**55/61 (90.2%)**	**194/215 (90.2%)**	**228/249 (91.6%)**

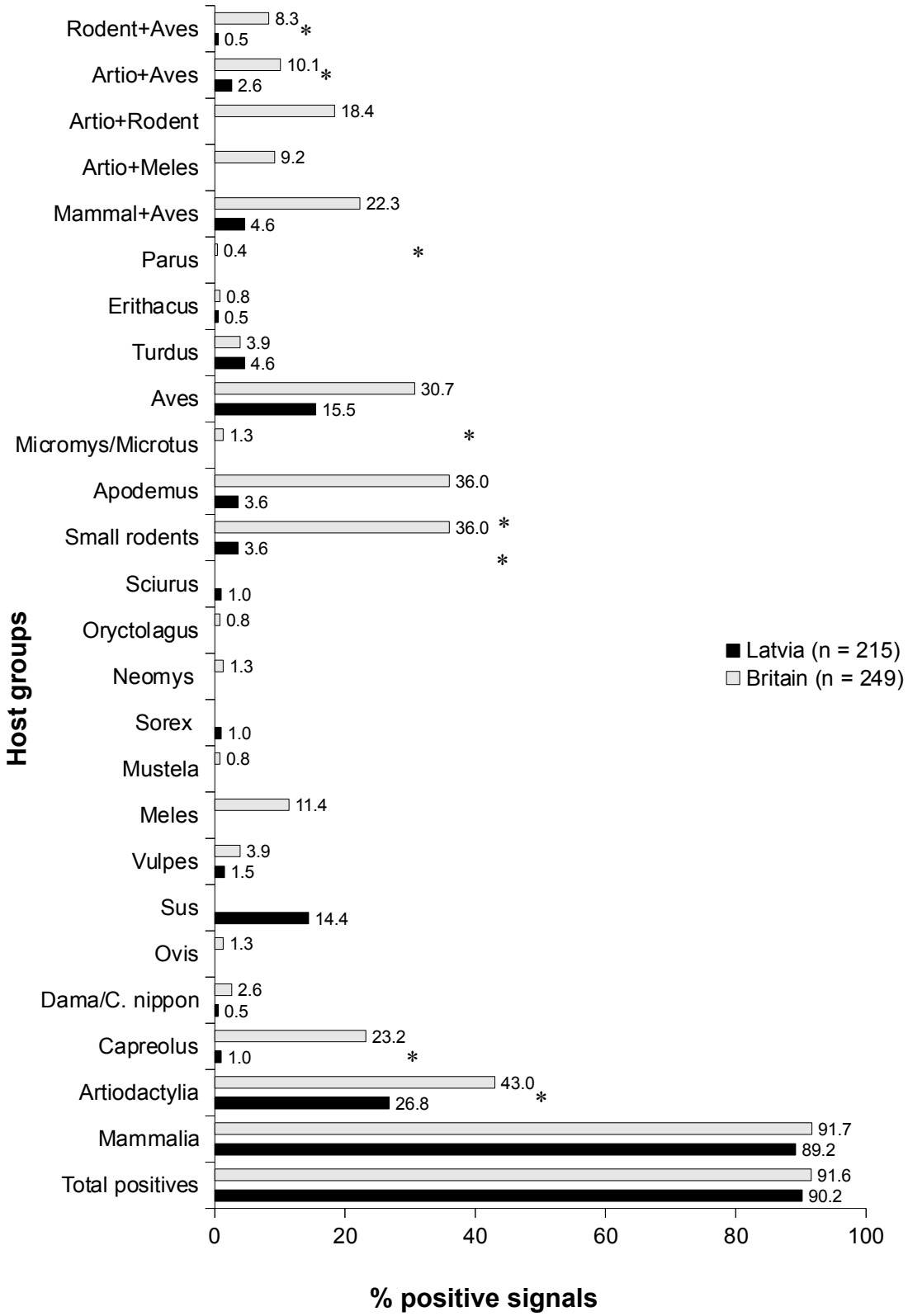


Fig. 5.5. Bar chart of percentages of positive signals found in all ticks (nymphs and adults) from Latvia and Britain for 20 RLB oligo probes and combinations of double positive signals. Significantly different ($P < 0.05$) values between the two countries are denoted by asterisks.

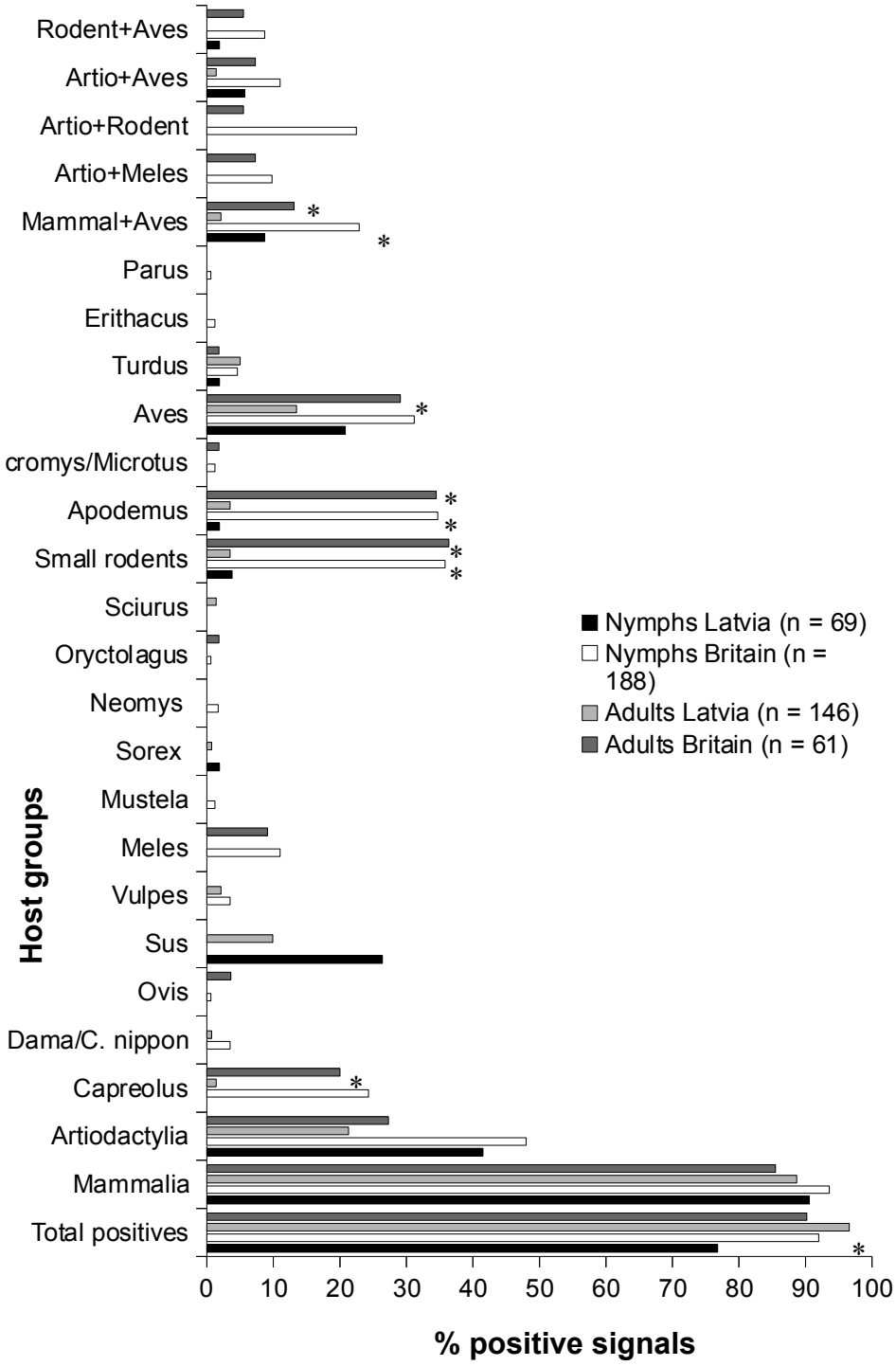


Fig. 5.6. Bar chart of percentages of positive signals found in nymphal and adult ticks from Latvia and Britain for 20 RLB oligo probes and combinations of double positive signals. Significantly different ($P < 0.05$) values between the two countries are denoted by asterisks.

5.2.1.2 Comparison of borrelia-negative and borrelia-positive ticks

Differentiation between borrelia-positive and negative ticks in the two populations showed significant differences for several host groups, with borrelia-positive ticks in most cases exhibiting higher rates of positive signals than their borrelia-negative counterparts. In this section, both borrelia-positive and negative ticks from each country (e.g. borrelia-positive vs. negative ticks from Latvia) were compared as well as the respective groups between the two countries (e.g. borrelia-positive Latvian vs. British ticks). The finding are summarized in Table 5.6.

In Latvian samples borrelia-negative ticks showed a significantly higher overall rate of positive signals, whereas borrelia-positive ticks showed a significantly higher proportion of positive signals for the artiodactyl probe, for wild boar (*Sus scrofa*), and for birds.

British borrelia-positive ticks displayed a significantly higher percentage of positive signals for the mammalian probe, for both artiodactyls in general and for roe deer (*C. capreolus*) in particular. They also exhibited a significantly higher rate of hybridization with the probes for small rodents, *Apodemus* spp., and thrushes (*Turdus* spp.), whereas borrelia-negative ticks showed a non-significantly higher rate of binding to the bird probe. borrelia-positive ticks were furthermore found to have significantly higher rates of double positive signals for artiodactyl+rodent and for artiodactyl+bird probes, respectively.

Both borrelia-positive and negative ticks from Britain reacted to a significantly higher extent with the probes for artiodactyls, roe deer, small rodents, and for *Apodemus* mice than their respective Latvian counterparts. British borrelia-negative ticks were furthermore binding more frequently to the bird probe than Latvian ticks, albeit showing a lower rate of overall positive signals. This observation was reversed for borrelia-positive ticks, where ticks from Britain displayed a higher rate than Latvian ticks.

Table 5.6. Proportions of borrelia-negative and borrelia-positive ticks from Latvia and Britain showing positive signals for one or more of 20 RLB oligo probes. The most important host groups are shown in bold. Superscript letters denote significant differences ($P < 0.05$, Fisher's exact test) between positive and negative ticks from each country **(a)**, or between the two countries **(b)**, respectively.

Host DNA identification	Latvian ticks		British ticks	
	borrelia- (n = 145)	borrelia+ (n = 70)	borrelia- (n = 177)	borrelia+ (n = 72)
Mammalia	**125/142 (88.0%)**	**48/52 (92.3%)**	**143/160 (89.4%)^{a}**	**66/68 (97.1%)^{a}**
Artiodactyla	**26/142 (18.3%)$^{a/b}$**	**26/52 (50.0%)$^{a/b}$**	**51/160 (31.9%)$^{a/b}$**	**47/68 (69.1%)$^{a/b}$**
Capreolus	1/142 (0.7%)^{b}	1/52 (1.9%)^{b}	15/160 (9.4%)$^{a/b}$	38/68 (55.9%)$^{a/b}$
Dama/ C. nippon	1/142 (0.7%)	-	-	6/68 (8.8%)
Ovis	-	-	3/160 (1.9%)	-
Sus	14/142 (9.9%)^{a}	14/52 (26.9%)^{a}	-	-
Vulpes	3/142 (2.1%)	-	-	6/68 (8.8%)
Meles	-	-	15/160 (9.4%)	9/68 (13.2%)
Mustela	-	-	-	2/68 (2.9%)
Sorex	1/142 (0.7%)	1/52 (1.9%)	-	-
Neomys	-	-	-	8/68 (11.8%)
Oryctolagus	-	-	1/160 (0.6%)	1/68 (1.5%)
Sciurus	2/142 (1.4%)	-	-	-
Small rodents	**6/142 (4.2%)^{b}**	**1/52 (1.9%)^{b}**	**44/160 (27.5%)$^{a/b}$**	**39/68 (57.4%)$^{a/b}$**
Apodemus	6/142 (4.2%)^{b}	1/52 (1.9%)^{b}	43/160 (26.9%)$^{a/b}$	39/68 (57.4%)$^{a/b}$
Micromys/ Microtus	-	-	-	3/68 (4.4%)
Aves	**17/142 (12.0%)$^{a/b}$**	**13/52 (25.0%)^{a}**	**53/160 (33.1%)^{b}**	**17/68 (25.0%)**
Turdus	5/142 (3.5%)	4/52 (7.7%)	3/160 (1.9%)^{a}	6/68 (8.8%)^{a}
Erithacus	-	1/52 (1.9%)	2/160 (1.3%)	-
Parus	-	-	1/160 (0.6%)	-
Mammal+ Aves	-	9/52 (17.3%)	36/160 (22.5%)	15/68 (22.1%)
Artio+ Meles	-	-	13/160 (8.1%)	8/68 (11.8%)
Artio+ Rodent	-	-	8/160 (5.0%)^{a}	34/68 (50.0%)^{a}
Artio+ Aves	-	5/52 (9.6%)	10/160 (6.3%)^{a}	13/68 (19.1%)^{a}
Rodent+ Aves	-	1/52 (1.9%)^{b}	10/160 (6.3%)	9/68 (13.2%)^{b}
Total positives	**142/145 (97.9%)$^{a/b}$**	**52/70 (74.3%)$^{a/b}$**	**160/177 (90.4%)^{b}**	**68/72 (94.4%)^{b}**

A comparison of the borrelia-positive ticks according the respective borrelia genospecies found within them revealed further differences between the two countries, as shown in Table 5.7. The genospecies of 61 of the 70 borrelia-positive ticks from Latvia could be identified. These ticks were further differentiated into nymphs and adult ticks. Six Latvian adult ticks (9.8%) could be included, compared to only three British adults (4.2%). Significantly lower proportions of the Latvian samples reacted with the probes for mammals (68.8%), artiodactyls (39.3%), and only 1.6% bound to the probe for roe deer, compared with 52.8% of the British samples. No positive signals at all could be identified from the probes for small rodents and for *Apodemus* mice. The prevalence of positive signals for birds and for other hosts (mostly badger, fox, and in case of Latvian samples, wild boar) was roughly equal between the two groups.

B. afzelii was present in 16 of 61 (26.2%) ticks from Latvia (Table 5.7A), and in 12 out of 72 British ticks (Table 5.7B). The British samples showed overall more positive signals for any host group or species, while signals from other hosts accounted for one quarter in both groups.

Both groups showed similar levels of *B. garinii* positives (about 40%), but of these, the British samples yielded a significantly higher rate of positive signals for artiodactyls while the proportion of positive signals for the bird probe reached about 20% in both groups. Other hosts accounted for 17.2% in the British and for 25.0% in the Latvian samples, respectively. British ticks tested positive for *B. valaisiana* displayed to a higher degree positive signals for the mammalian and artiodactyl probes, while the Latvian samples showed slightly higher frequencies of positive signals from birds and from other hosts. The overall numbers of mixed borrelia infections did not differ between the two countries, but British samples reacted significantly more often (100%) with the mammalian probe than ticks from Latvia (40.0%).

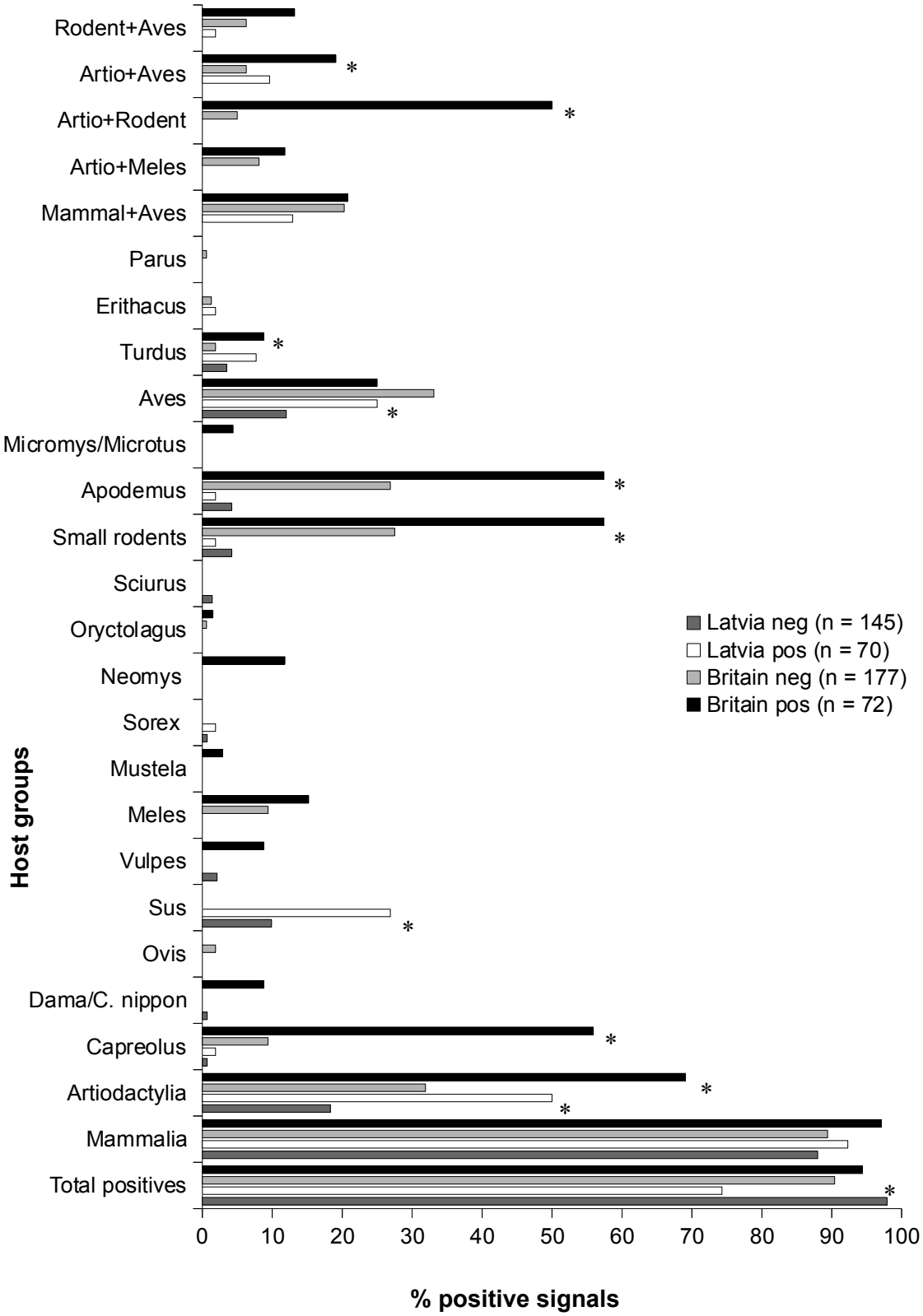


Fig. 5.7. Bar chart of percentages of positive RLB signals found in borrelia-positive and borrelia-negative ticks (nymphs and adults) from Britain and Latvia for 20 RLB oligo probes and combinations of double positive signals. Values that are significantly different ($P < 0.05$) between borrelia-positive and negative ticks from each country are denoted by asterisks.

Table 5.7. Distribution and frequency of positive host signals found in ticks tested positive for different borrelia genospecies, (A) from Latvia, and (B) from Britain. Total distributions are shown in the top row of each group, and separated into nymphs (n) and adults (a) below. Values that are significantly different ($P < 0.05$) between Latvia and Britain are denoted by asterisks.
(A):

Host DNA identification	Ticks tested positive for borrelia genospecies									
	B. afzelii 16/61 (26.2%)		***B. garinii*** 24/61 (39.3%)		***B. valaisiana*** 16/61 (26.2%)		**Mixed borrelia species** 5/61 (8.2%)		**Total (61)**	
	n	**a**	**n**	**a**	**n**	**a**	**n**	**a**	**n**	**a**
	16/16 (100%)	-	**21/24 (87.5%)**	**3/24 (12.5%)**	**15/16 (93.8%)**	**1/16 (6.3%)**	**3/5 (60.0%)**	**2/5 (40.0%)**	**55/61 (90.2%)**	**6/61 (9.8%)**
Mammal	10/16 (62.5%)		18/24 (75.0%)		12/16 (75.0%)		2/5 (40.0%)*		**42/61 (68.8%)***	
	10/16 (62.5%)	-	15/24 (62.5%)	3/24 (12.5)	11/16 (68.8%)	1/16 (6.3%)	1/5 (20.0%)*	1/5 (20.0%)	**37/61 (60.7%)***	**5/61 (8.2%)**
Artiodactyls	5/16 (31.3%)		9/24 (37.5%)*		9/16 (56.3%)		1/5 (20.0%)		**24/61 (39.3%)***	
	5/16 (31.3%)	-	7/24 (29.2%)	2/24 (8.3%)	8/16 (50.0%)	1/16 (6.3%)	-	1/5 (20.0%)	**20/61 (32.8%)***	**4/61 (6.6%)**
Capreolus	-		-		-		1/5 (20.0%)		**1/61 (1.6%)***	
	-	-	-	-	-	-	-	1/5 (20.0%)	-	**1/61 (1.6%)**
Small rodents	-		-		-		-		-	
	-	-	-	-	-	-	-	-	-	-
Apodemus	-		-		-		-		-	
	-	-	-	-	-	-	-	-	-	-
Birds	1/16 (6.3%)		5/24 (20.8%)		2/16 (12.5%)		1/5 (20.0%)		**9/61 (14.8%)**	
	1/16 (6.3%)	-	4/24 (16.7%)	1/24 (4.2%)	2/16 (12.5%)	-	-	1/5 (20.0%)	**7/61 (11.5%)**	**2/61 (3.3%)**
Other hosts	4/16 (25.0%)		6/24 (25.0%)		4/16 (25.0%)		-		**14/61 (23.0%)**	
	4/16 (25.0%)	-	5/24 (20.8%)	1/24 (4.2%)	4/16 (25.0%)	-	-	-	**13/61 (21.3%)**	**1/61 (1.6%)**

Table 5.7. (continued)
(B):

Host DNA identification	Ticks tested positive for borrelia genospecies									
	B. afzelii 12/72 (16.7%)		***B. garinii*** 29/72 (40.3%)		***B. valaisiana*** 22/72 (30.6%)		**Mixed borrelia species** 9/72 (12.5%)		**Total (72)**	
	n	**a**	**n**	**a**	**n**	**a**	**n**	**a**	**n**	**a**
	11/12 (91.7%)	**-**	**26/29 (89.7%)**	**-**	**19/22 (86.4%)**	**3/22 (13.6%)**	**9/9 (100%)**	**-**	**69/72 (95.8%)**	**3/72 (4.2%)**
Mammal	11/12 (91.7%)		26/29 (89.7%)		20/22 (90.0%)		9/9 (100%)*		**66/72 (91.7%)***	
	11/12 (91.7%)	-	26/29 (89.7%)	-	17/22 (77.3%)	3/22 (13.6%)	9/9 (100%)	-	**63/72 (87.5%)***	**3/72 (4.2%)**
Artiodactyls	7/12 (58.3%)		23/29 (79.3%)*		14/22 (63.6%)		3/9 (33.3%)		**47/72 (65.3%)***	
	7/12 (58.3%)	-	23/29 (79.3%)	-	12/22 (54.5%)	2/22 (9.1%)	3/9 (33.3%)	-	**45/72 (62.5%)***	**2/72 (2.8%)**
Capreolus	4/12 (33.3%)		20/29 (69.0%)		11/22 (50.0%)		3/9 (33.3%)		**38/72 (52.8%)***	
	4/12 (33.3%)	-	20/29 (69.0%)	-	9/22 (40.9%)	2/22 (9.1%)	3/9 (33.3%)	-	**36/72* (50.0%)**	**2/72 (2.8%)**
Small rodents	5/12 (41.7%)		19/29 (65.5%)		11/22 (50.0%)		4/9 (44.4%)		**39/72 (54.2%)**	
	5/12 (41.7%)	-	19/29 (65.5%)	-	8/22 (36.4%)	3/22 (13.6%)	4/9 (44.4%)	-	**36/72 (50.0%)**	**3/72 (4.2%)**
Apodemus	5/12 (41.7%)		19/29 (65.5%)		11/22 (50.0%)		4/9 (44.4%)		**39/72 (54.2%)**	
	5/12 (41.7%)	-	19/29 (65.5%)	-	8/22 (36.4%)	3/22 (13.6%)	4/9 (44.4%)	-	**36/72 (50.0%)**	**3/72 (4.2%)**
Birds	3/12 (25.0%)		6/29 (20.7%)		5/22 (22.7%)		2/9 (22.2%)		**13/72 (18.1%)**	
	3/12 (25.0%)	-	6/29 (20.7%)	-	5/22 (22.7%)	-	2/9 (22.2%)	-	**13/72 (18.1%)**	**-**
Other hosts	3/12 (25.0%)		5/29 (17.2%)		4/22 (18.2%)		2/9 (22.2%)		**13/72 (18.1%)**	
	3/12 (25.0%)	-	5/29 (17.2%)	-	4/22 (18.2%)	-	2/9 (22.2%)	-	**13/72 (18.1%)**	**-**

5.3 Discussion

5.3.1 Comparison of ticks from BW and RW

5.3.1.1 Overall comparison between BW and RW

This study is the first attempt to assess the feeding preferences of *I. ricinus* ticks from habitats in Southwest England on a molecular basis. It could be shown that ticks in two habitats that were only a short distance (ca. 2 km) apart exhibit significant differences in diversity and prevalence of vertebrate hosts (see Table 5.2, Figures 5.2 and 5.3). In the previous chapter it has been shown that RW harbours a greater diversity of host animal

species than BW, probably due to the ecotonal fragmentation of its habitat. This is also reflected in the observation that ticks in RW had fed on a broader range of hosts, including larger mammals like sheep, foxes, weasels and rabbits, but also on field voles or harvest mice. They also fed significantly more frequently on birds than ticks from BW, which in turn showed a higher prevalence for mammals. This could be explained by the denser and richer vegetation in RW, which might provide better cover both for questing ticks and for foraging animals that could act as hosts. In a study from Switzerland, Moran Cadenas et al. (2007) employed a similar molecular technique to compare blood meals from ticks sampled from the north- and south facing slopes of a mountain. They observed similar distributions of the different host groups, with artiodactyls accounting for about 40-50% of all positive signals, followed by carnivores, birds and red squirrels, while small rodents only contributed about 10% of the positive signals in both sites. While the rate of rodent signals was higher in the observations from Bath, it was also found that ticks on a north facing, sloped site dominated by coniferous trees (similar to BW) fed less frequently on birds than on a south facing site. It would be desirable to be able to predict tick feeding behaviour in response to the availability of host species, such as the 'forage ratio' described by Hess et al. (1968, cited in Kent, 2009) or the 'feeding index' proposed by Kay et al. (1979, cited in Kent, 2009). It is, however, vastly more difficult to obtain the necessary quantitative data on host abundance (see chapter 4.2 of this thesis) for a parasite with such a catholic host spectrum as *I. ricinus* than for more specialised arthropods, such as some mosquito species (Kilpatrick et al., 2006).

Apart from the fact that small rodents accounted only for about one third of all blood meals of larval ticks (which were subsequently detected in questing nymphs after the larval-nymphal moult), the role of artiodactyls as major hosts for larval ticks should be highlighted. Several animal species that are commonly assumed to be important hosts for *I. ricinus* were notably absent in the findings of this study. These include bank voles (*Myodes glareolus*, see e.g. Talleklint & Jaenson, 1995; 1997), squirrels (*Sciurus* spp., see Craine et al., 1997), brown hares (*Lepus europaeus*, see Talleklint & Jaenson, 1993), hedgehogs (*Erinaceus europaeus*, see Gray et al., 1994) and common pheasants (*Phasianus colchicus*, see Kurtenbach et al., 1998). No brown hares or pheasants, and only very few hedgehogs were observed at the two study sites (see chapter 4 results section), which was confirmed by their absence as tick hosts in the blood-meal analysis. Grey squirrels (*S. carolinensis*)

on the other hand were very frequently seen in both sites, both in the tree canopy and feeding on the ground. The oligo probe for squirrels employed in this study was designed by Humair et al. (2007) according to the published 12S DNA sequence for the red squirrel (*S. vulgaris*), which is common in mainland Europe, but absent from most habitats in Southern England, where it has been replaced by the grey squirrel (Gurnell & Hare, 2008). It seems likely that the oligo probe was too specific to identify DNA from a different species of the same genus that had developed on a different continent. No 12S DNA sequence of grey squirrels has been published so far, thereby preventing the design of a more specific oligo probe. It has previously been demonstrated that bank voles showed much lower infestation rates with *I. ricinus* than wood mice (e.g. Kurtenbach et al., 1995; Hanincova et al., 2003, also see chapter 4 of this thesis), but their complete absence in the blood-meal analysis appears nevertheless surprising. Bank voles acquire immune resistance against ticks (Dizij & Kurtenbach, 1995), which is also affected by their testosterone levels (Hughes & Randolph, 2001), and which might explain why ticks analysed in this study avoided them as hosts.

Interestingly, ticks from both sites exhibited a high percentage (ca. 20%) of double positive signals for both mammal and bird probes, indicating that the feeding process on a first host animal is quite frequently interrupted and has to be resumed on a new host. A similar rate of double positive signals has been described by Moran Cadenas et al. (2007), who also suggested that failed feeding attempts might lead to contamination of the ticks with host DNA. Furthermore, ticks from RW showed higher rates of double positive signals for artiodactyls+small rodents, artiodactyls+birds, and for small rodents+birds. These findings illustrate the fact that the feeding process of *I. ricinus* ticks happens in a less straightforward manner than previously assumed, and that repeated attempts are often necessary until a full blood-meal might be obtained by a tick. This could also have implications for the ecology of pathogens that might be transmitted between different hosts, such as *B. burgdorferi* s.l. spirochetes (see next section).

5.3.1.2 Comparison of borrelia-negative and borrelia-positive ticks

When the ticks from BW and RW were differentiated between borrelia-negative and borrelia-positive ticks it emerged that positive ticks from BW had significantly higher prevalences of positive signals for artiodactyls, roe deer and small rodents than negative ticks, while positive ticks from RW displayed significantly more positive signals for

mammals, artiodactyls and roe deer, but only a non-significant increase of small rodent signals. Although non-significant, borrelia-positive ticks from both sites exhibited a reduced frequency of positive signals from birds compared to borrelia-negative ticks (see Table 5.3).

It has previously been observed that infections can alter the behaviour of *Ixodes* ticks. Randolph (1991) showed that infections with *Babesia microti* improved the feeding success and survival in *I. trianguliceps*, which was also confirmed for *I. scapularis* in North America (Hu et al., 1997). The effects of infections with *B. burgdorferi* differed between the tick stages: while *I. ricinus* nymphs infected with *B. burgdorferi* s.l. showed increased activity under dry conditions (Perret 2003, cited in Gassner, 2010), and infection with *B. burgdorferi* s.s. led to increased phototaxis and vertical activity in *I. scapularis* nymphs, adult *I. scapularis* exhibited decreased levels of activity (Lefcort & Durden, 1996). Lane et al. (2007) could demonstrate that *B. burgdorferi* s.s. infected nymphs of *I. pacificus* quested higher in the vegetation than uninfected nymphs, which could explain why ticks would feed on larger animals, such as deer, but would not provide an explanation for the higher frequencies of mice as hosts. These findings reflect a changed balance in the activity patterns of infected ticks, leading to a more aggressive host seeking behaviour. This would ensure both the tick survival and transmission of the pathogen, while at the same time enduring higher physiological stress due to desiccation and energy consumption. Interestingly, the shift from birds towards mice and artiodactyls observed in borrelia-infected larvae and nymphs (as seen in their subsequent questing stages used for the BMA) observed in this study would not favour the survival of bird-associated borrelia genospecies that are more prevalent in Southwest England (Kurtenbach et al., 1998; Vollmer et al., 2011), as these spirochetes would be attacked by the complement system of incompetent hosts, such as wood mice (Kurtenbach et al., 1998; 2002) or roe deer (Jaenson & Talleklint, 1992). This might be offset by the increased survival rate of ticks feeding on larger hosts such as deer. It should also be noted that signals of sika deer (*Cervus nippon*) were found in 17% of the borrelia-positive ticks from RW. Unlike other deer species, both roe deer and sika deer have been shown to harbour *B. burgdorferi* s.l. spirochaetes in their skin (Kimura et al., 1995; Pichon et al., 2000), thereby indicating that a broader host spectrum might support enzootic borrelia cycles. Although they showed lower rates of certain positive signals, it is also evident that the borrelia-negative ticks must have fed

successfully on a host while in their previous stage, since they would not have moulted otherwise. This raises the issue whether other host species that have not been identified yet could have provided these blood meals. One of the more severe limitations of the BMA technique employed in this study is the fact that, despite the utmost care, contamination of the samples with human DNA was virtually impossible to avoid. As described in section 2.2.6.1, each step of the analysis was carried out in separate areas that were regularly swiped with dH_2O and treated with UV light to reduce the risk of DNA contamination. Nevertheless, it appears impossible to avoid contamination with human DNA under the current protocol. Therefore the oligo probe for human hosts had to be excluded from the analysis, as described by Humair et al. (2007).

The absence of a strict association between different borrelia genospecies and prominent host groups (as seen in Table 5.4) has been observed in previous studies (Pichon et al., 2003; 2005; Estrada-Peña et al., 2005; Moran Cadenas et al., 2007). This might be explained by the possibility that some animal species considered incompetent hosts (such as deer) for some or all borrelia genospecies could in reality offer some degree of reservoir competence. Co-feeding of ticks, during which transmission of borrelia might occur in the absence of a systemic infection of the host (Hu et al., 2003), has previously been described for artiodactyl hosts such as sheep (Ogden et al., 1997) and sika deer (Kimura et al., 1995), and transovarial transmission have also been put forward as possible explanations (Moran Cadenas et al., 2007). It is important to note, however, that the detection of borrelia DNA by PCR during the screening of ticks does not necessary indicate the presence of viable spirochaetes in the ticks, as the bacteria might have been killed by the complement arm of the host's immune system that provided the blood meal for the tick.

5.3.2 Comparison between Latvian and British populations

When comparing the analysed ticks from Latvia and Britain (see Table 5.5), similar levels of mammalian positive signals were found, but significantly more British samples displayed positive signals for artiodactyls, roe deer, and especially for small rodents, including *Apodemus* mice. The rates for positive bird signals were comparable for nymphs (that had fed as larvae), but higher for British adult ticks. The absence of positive signals for specific hosts in many of the Latvian samples could either be explained by failed hybridization, as experienced in about 50% of analysed ticks in the study by Humair et al. (2007), or potentially indicate that immature stages of *I. ricinus* from the Latvian

populations had previously fed on other host species that have not been tested in this assay but are present in the area. These include the European elk (*Alces alces* L.), carnivores such as stone marten (*Martes foina* Erxleben) and pine marten (*M. martes* L.), American mink (*Neovison vison* [syn. *Mustela vison*] Schreber), raccoon dog (*Nyctereutes procyonides* Gray), and aquatic rodents, such as European water vole (*Arvicola amphibius* [syn. *A. terrestris*] L.), muskrat (*Ondatra zibethicus* L.) and Eurasian beaver (*Castor fiber* L.). Further small rodent species encountered in the Latvian habitats were the pygmy field mouse (*Apodemus uralensis* Pallas), the striped field mouse (*Apodemus agrarius* Pallas), and the sibling vole (*Microtus levis* [syn. *M. rossiaemeridionalis*] Miller) (see Ozolins & Pilats, 1995; Zorenko & Leontyeva, 2003).

Humair et al. (2007) observed a lower rate of positive signals from ticks that had been collected in spring than in ticks collected in autumn in a previous study (Pichon et al., 2005). It was suggested by Humair et al. (2007) that ticks that had fed late in the previous activity season (in late summer/autumn of the previous year) would provide the best chance of host detection. These ticks would then enter developmental diapause and would not continue to develop further until mid-summer of the following year, when they would be questing again. This, together with the fact that most of the Latvian ticks had been collected in 2002, whereas all British ticks were from 2006 and 2007, might help to explain their reduced rate of positive signals.

The 12S rRNA target gene used in this study was chosen by Humair et al. (2007) because it offered less inter- and intraspecific variability than the mitochondrial *cytochrome b* gene employed previously (e.g. Kirstein & Gray, 1996), but was less rigidly conserved that the nuclear 18S rRNA gene used in earlier studies (Estrada-Peña et al., 2005; Pichon et al., 2003; 2005). The very small amount of positive signals for bird species in this study, despite higher rates for the bird group probe, invites speculation, however, whether these non-degenerate primers might be too specific and would not allow for intra-specific variation within these bird species.

As described above (section 5.3.1.1), the limited amount of quantifiable data on host abundance in the habitats of both Latvia and Britain would make a more specific prediction of host preferences extremely difficult (see Kent, 2009). The observation that British ticks showed rates of positive signals for badgers comparable with those of Latvian ticks for wild boar might on the other hand indicate that in the absence of one host (wild boar in

most of Britain, see Putman, 2008) another host with a comparable biology, such as badgers, would be utilised by *I. ricinus* ticks.

As described in section 5.3.1.2 for the British ticks, a comparison of borrelia-negative and positive ticks from both countries revealed significant increases in positive signals for some host groups (such as mammals and artiodactyls), while signals for small rodents were more frequent in borrelia-negative than in positive Latvian ticks (see Table 5.6). Furthermore, as shown in Table 5.7, a strong discrepancy was observed between borrelia genospecies and host signals found in Latvian ticks. As previously mentioned, a PCR signal of a certain borrelia genospecies in a tick would not necessarily imply viable spirochetes in the tick, but merely the presence of genospecific borrelia DNA. Given the extent of double positive signals for different host species or groups, repeated feeding attempts on different hosts would offer another explanation (see Moran Cadenas et al., 2007).

5.4 Conclusions

Apart from the possible need to optimise of some of the oligo probes to take intra-specific variation into account, the BMA technique employed in this assay allows for a detailed description of host feeding behaviour of individual ticks on a molecular level. It could be shown that British ticks exhibited differences in host utilisation on a fine-scale geographical level between two different habitats near Bath, UK. Repeated feeding attempts, resulting in mixed host signals, appear to occur much more commonly than previously thought. Infection with borrelia spirochaetes increased the rates of positive host signals significantly. British ticks showed higher rates of positive signals than ticks from Latvia, possibly due to lower host DNA quality in the Latvian samples, or a local host fauna not covered sufficiently by the probes used in this study.

6. Endobacterial fauna of *I. ricinus* tick populations

6.1 Introduction

6.1.1. *Wolbachia* is a global endosymbiont of arthropods that can alter their phenotypes

Wolbachia are a group of endobacteria that belong to the order Rickettsiales, a diverse group of α-Proteobacteria with an obligate intracellular lifestyle that comprises mutualistic, commensal and parasitic species. *Wolbachia* are found in arthropods (including Acari) and nematodes and are transmitted vertically through the eggs of the hosts (Werren et al., 2008). They are also able to move horizontally between different arthropod species and are one of the most common bacteria groups with a very diverse and global distribution, infecting about 20-75% of all arthropod species (reviewed by Hilgenboecker et al., 2008). *Wolbachia* are closely related to pathogen genera such as *Anaplasma*, *Ehrlichia* and *Rickettsia* that are transmitted by ticks and are commonly associated with vertebrate hosts, whereas *Wolbachia* do not infect vertebrates (Werren et al., 2008). Several different *Wolbachia* species have been described, including *W. persica* (Suitor & Weiss, 1961) in *Argas persicus* (Oken, 1818) ticks, and *W. pipientis*, which is regarded as the type species of the group, and was discovered in cells of the mosquito *Culex pipiens* in 1924 (Hertig & Wolbach, cited in Lo et al., 2007). All *Wolbachia* endosymbionts are grouped in eight supergroups (A-H), based on their 16S rDNA sequences (Casiraghi et al., 2005; Baldo & Werren, 2007). Due to taxonomic uncertainty, it has been proposed to name all endosymbionts clustering closely with the supergroups as *W. pipientis* (Lo et al., 2007), while *W. persica* has been suggested to belong to a different clade within the γ-Proteobacteria (O'Neill et al., 1992). In the following text these endosymbionts are referred to by their generic name *Wolbachia*.

Wolbachia infections are capable of inducing resistance in *Drosophila* and mosquitos against RNA viruses (Teixeira et al., 2008), such as Dengue (Bian et al., 2010), Chikungunya (Moreira et al., 2009), West Nile (WNV) (Glaser & Meola, 2010) and against the human malaria parasite *Plasmodium falciparum* (Kambris et al., 2010; Hughes et al., 2011).

Wolbachia have been shown to manipulate the sex ratio of their host populations by inducing four different phenotypes. Two of these phenotypes are found in Acari: (1) cytoplasmic incompatibility (CI), which prevent males from successfully mating with

female individuals that do not possess the same strain of *Wolbachia*, and (2) parthenogenesis, in which males are eliminated from reproduction. Other sex-changing phenotypes observed in *Wolbachia*-infected insects, but not Acari include the changing of males into females (feminization), and the killing of infected males, while their infected female siblings survive (reviewed by Werren et al., 2008). The occurrence of a *Wolbachia*-induced sex bias in *I. ricinus* could have serious implications for the epidemiology of infectious diseases caused by pathogens transmitted by these ticks, such as Lyme borreliosis (LB) or human granulocytic anaplasmosis (HGA). While immature stages of both sexes require blood meals, only the adult females, but not the male ticks feed on vertebrate hosts (Hillyard, 1996). Therefore a higher ratio of females would increase the density of adult females that would be capable of transmitting pathogens while feeding. Female *I. ricinus* have also been observed to be more philopatric than males (de Meeus et al., 2002), while the two sexes were shown to possess differential prevalence rates of LB spirochaetes (de Meeus et al., 2004). A simple approach was developed in our group by which tick samples could be tested by PCR for signals of *Wolbachia.*

6.1.2 *Midichloria* is a unique endosymbiont of mitochondria in hard ticks

Midichloria are bacterial endosymbionts that were originally found in *I. ricinus* (first described by Lewis, 1979; later identified through 16S rDNA analysis by Beninati et al., 2004; and morphologically by Sacchi et al., 2004) and possess the unique ability to invade mitochondria in the host cells (Sacchi et al., 2004). They belong to a novel clade within the order Rickettsiales (α-Proteobacteria) and have been named '*Candidatus* Midichloria mitochondrii' (in the following text referred to as *Midichloria*) (Sassera et al., 2006). In *I. ricinus* samples from different European countries, male ticks were found to be infected at a lower rate (44%) than female ticks, which were all infected (Lo et al., 2006; Sassera et al., 2006). These endosymbionts reside mainly within the mitochondria of the female reproductive tract, from where they are vertically transmitted to the tick offspring (Beninati et al., 2004; Lo et al., 2006). However, apart from this maternal transfer, a horizontal transmission via co-feeding between ticks has also been suggested, similar to transmission routes of LB spirochaetes (Epis et al., 2008).

Since their discovery in *I. ricinus*, *Midichloria* have also been found through PCR in several other ticks species from various genera (Epis et al., 2008; Parola et al., 2003; Venzal et al., 2008), but are notably absent in *I. holocyclus* from Australia (Beninati et al.,

2009). *Midichloria* have also been found in the mitochondria of other blood-feeding arthropods, such as mites (Reeves et al., 2006, cited in Beninati et al., 2009), bed bugs (Richard et al., 2009, cited in Beninati et al., 2009), and horse flies (*Tabanus* spp.) (Hornok et al., 2008, cited in Beninati et al., 2009). Furthermore, closely related endosymbionts have recently also been detected in cell lines derived from various tick species (Najm et al., 2011). Apart from their existence in medically important arthropods, they do not seem to possess any clinical relevance themselves (Beninati et al., 2009).

Given these unique characteristics of *Midichloria*, it was attempted to establish a MLST scheme for this endosymbiont by testing several housekeeping genes on *I. ricinus* samples from various European countries.

The aims of the work carried out to support this chapter were:

- Test whether borrelia-positive and borrelia-negative ticks displayed different prevalence rates with the CI-inducing endosymbionts *Wolbachia*.
- Screen British and Latvian ticks for the prevalence of *Midichloria.*
- Test several *Midichloria* housekeeping genes in ticks from various European locations in order to reconstruct their phylogenetic relationships.

6.2 Results

6.2.1 *Wolbachia* prevalence in British *I. ricinus* ticks

A total of 176 adult ticks that had been collected in BW and RW in Britain in 2008 were tested for the presence of *Wolbachia* endobacteria. These samples were screened by PCR for a fragment of the *wsp* (*Wolbachia* surface protein) gene of approximately 590-630 bp length. Figs. 6.1 and 6.2 show the gel images of two batches of 88 tick samples each, respectively. Amplified clonal *wsp* DNA was used as positive control and was kindly provided by Jennifer Garbutt (University of Bath, UK). The primers employed were: wsp81-F and wsp-691-R, designed after Braig et al., 1998. In order to increase the intensity of the very weak bands in the agarose gels, several variations of the original PCR protocol were tested, such as varying annealing temperatures, changing the primer concentrations, reamplifiying the purified PCR products, or adding $MgCl_2$. However, no differences in the

strength of the bands could be observed (data not shown). Five positive PCR products from each group were sequenced and could be confirmed by BLAST analysis to be fragments of *wsp*.

Nineteen out of 176 samples (10.8%) were tested positive in the batch of British *I. ricinus* ticks. Due to time constraints no further samples could be tested for the presence of *Wolbachia*.

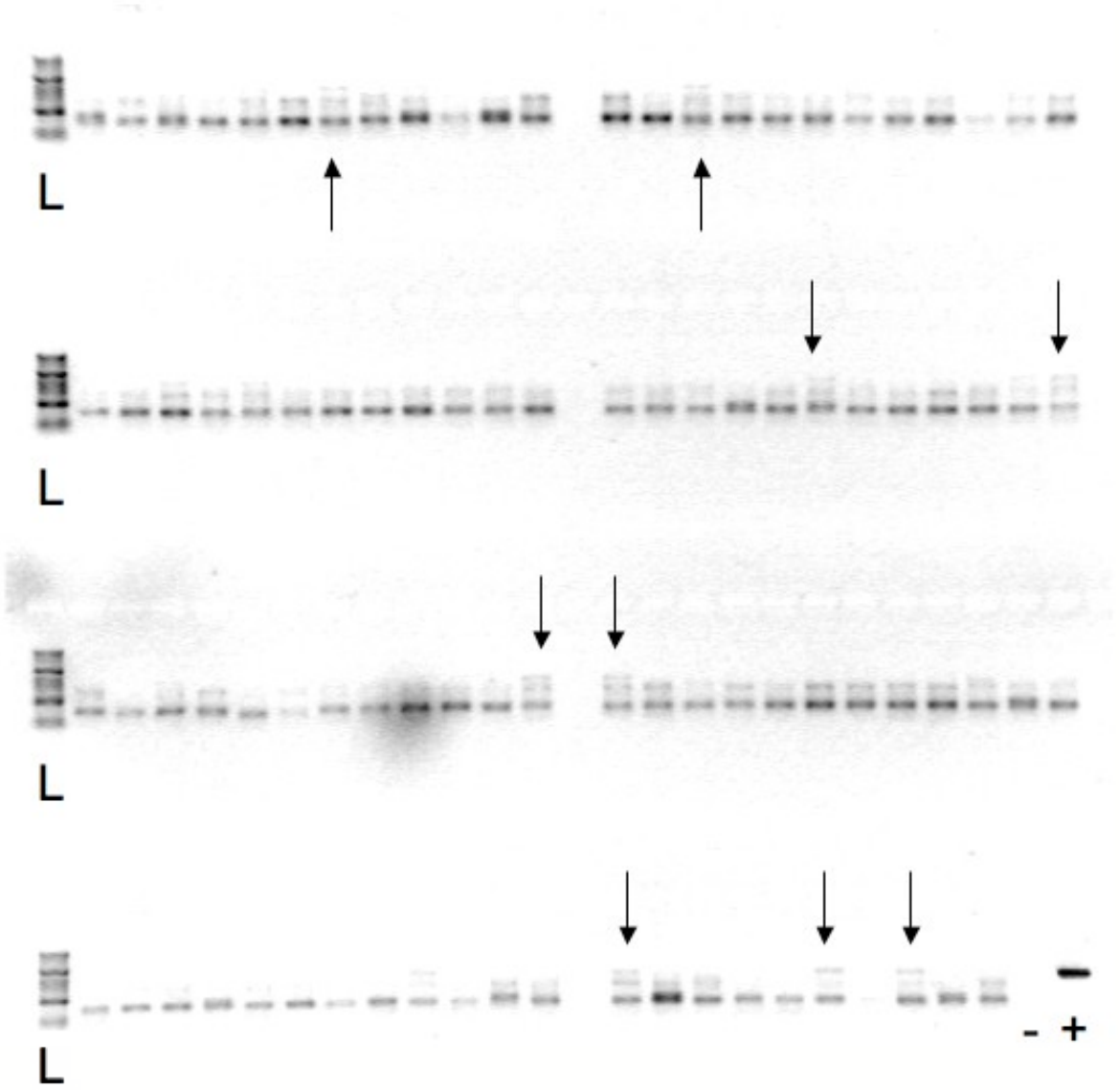


Fig. 6.1. Agarose gel image of 88 *I. ricinus* ticks from Britain tested for presence of a ca. 600 bp *wsp* sequence from *Wolbachia* sp. Arrows indicate faint bands at correct fragment length. L = gel ladder; + = *wsp* positive control; - = negative control.

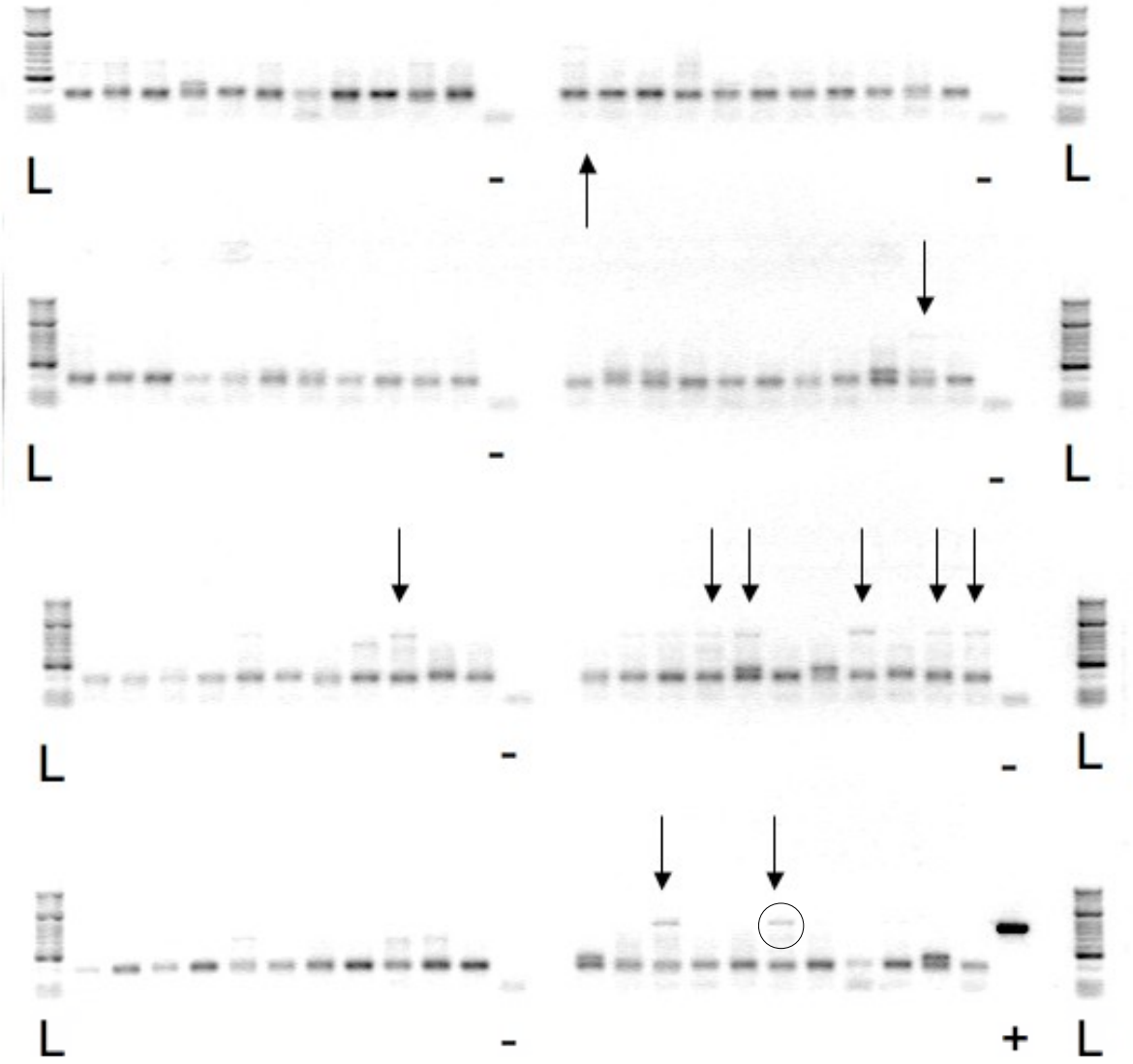


Fig. 6.2. Agarose gel image of 88 *I. ricinus* ticks from Britain tested for presence of a ca. 600 bp *wsp* sequence from *Wolbachia* sp. Arrows indicate faint bands at correct fragment length, see also circle. L = gel ladder; + = *wsp* positive control; - = negative control.

6.2.3 Prevalence of *Midichloria mitochondrii* endobacteria in British ticks

Forty adult *I. ricinus* ticks (20 males and 20 females) each from Latvia and from the UK were tested by Stavros Gavkalias for the presence of *Midichloria* endobacteria. This work formed part of an MRes research project and was carried out under my supervision. The tick samples were screened by PCR by using primers for a 390 bp-fragment of the 16S rDNA of *Midichloria*. These primers had been designed from the partial 16S rDNA sequence of *Midichloria* (GenBank accession no. AJ566640.2, published by Beninati et al.,

2004). All female ticks tested ticks showed a positive signal for the presence of *Midichloria*, whereas 14 out 20 British males, and 19 out 20 Latvian male ticks showed a positive signal (data not shown). This could further be confirmed by a BLAST analysis of the amplified sequences of eight (four from each country) randomly selected samples.

6.2.4 Phylogenetic analysis of *Midichloria* housekeeping gene sequences

Following the confirmation of *Midichloria* in our tick samples, primers for 12 candidate housekeeping genes that had been selected from *Bartonella* and *Orientia* were designed by Davide Sassera (University of Milan, Italy).

In order to assess the genetic diversity of *Midichloria* samples across the range of its host *I. ricinus* in Europe, 30 samples were selected. These included five adult ticks (both males and females) from each of the following six countries:

England, Scotland, Latvia, Germany, Switzerland, Portugal.

The ticks from England had been collected in BW and RW, and the Scottish ticks near Inverness, while the Latvian ticks had been collected in the sites in Babite, Kermeri, and Jaunciemis described previously. Appendix A denotes the geographic coordinates of all collection sites used in this study.

These findings were compared with the data obtained from the mtMLST scheme for *I. ricinus* developed by Ruth Dinnis.

The PCR amplification of all 12 genes in these tick samples was carried out by Stavros Gavkalias as part of his MRes research project, while all downstream data analysis was done by myself. Twenty-one out of 30 samples provided amplicons and could be included in the eventual analysis. The following five genes were chosen for an MLST scheme: *adk*, *lipA*, *nuoF*, *ppdK*, and *secY.* The sequences of the genes *lipB*, *gps*, *mdh*, *nrdB*, *sodB*, *sucA* and *sucD* did not reveal any nucleotide variations between the tested samples and were therefore excluded from the study. Appendix B lists the complete PCR conditions and primers employed in this study.

The obtained sequences of the five loci were concatenated to a sequence of 2376 bp length. A Neighbour-Joining tree with 1,000 bootstrap replicates is shown in Fig. 6.3.

Allelic profiling of the sequences led to six different sequence types (STs) in the tested population, and a subsequent cluster analysis carried out in eBURST (Feil et al., 2004)

revealed the statistical information displayed in Table 6.1. A graphic display of the clustering analysis was conducted by using goeBURST 1.2.1 (Francisco et al., 2009) and is depicted in Fig. 6.4. It shows the very low diversity of the *Midichloria* sequences, with 11 out of 21 samples that originated from Germany, Latvia, Portugal and Switzerland sharing an identical allelic profile (ST 1), and only one sample forming a singleton (ST 3) outside the clonal complex that contained the remaining 20 samples.

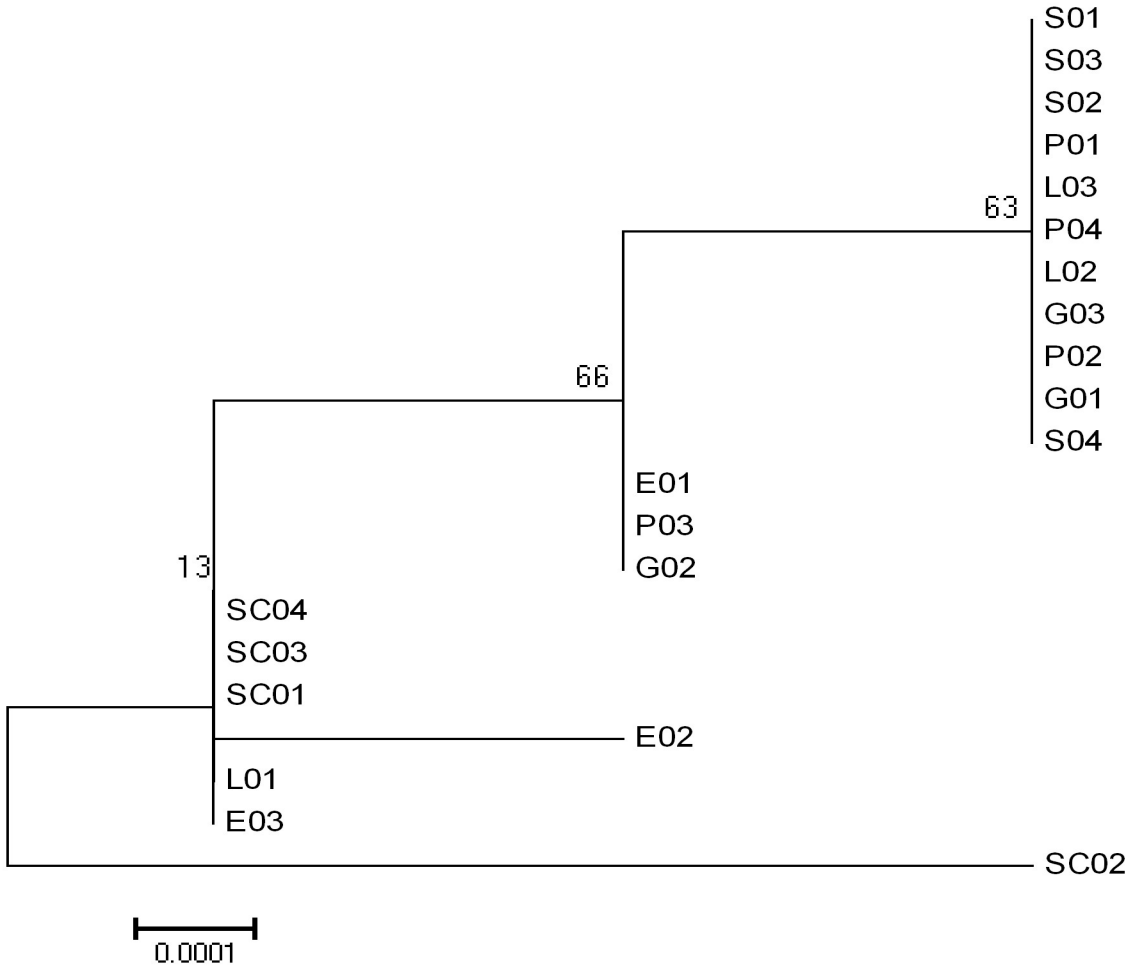


Fig. 6.3. Phylogenetic reconstruction inferred from the concatenated sequences of five housekeeping gene fragments (*adk*, *lipA*, *nuoF*, *ppdK*, and *secY*) of *Midichloria* endosymbionts from 21 *I. ricinus* ticks sampled in six different European countries using the Neighbour-Joining (NJ) method. The bootstrap consensus tree of 1000 repeats is shown. There were a total of 2376 positions in the final dataset. The scale bar represents 0.01% nucleotide difference. Bootstrap values are shown on the branch nodes. E: England; G: Germany; L: Latvia; P: Portugal; S: Switzerland; SC: Scotland.

Table 6.1. Statistics of eBURST analysis of 6 sequence types (STs) obtained from 21 *Midichloria* sequences. Countries of origin for the tick samples are shown. E: England; G: Germany; L: Latvia; P: Portugal; S: Switzerland; SC: Scotland. ST 6 was predicted as founder strain (indicated by superscript F), while ST 3 formed a singleton (indicated by asterisk). SLV: single locus variants; DLV: double locus variants; TLV: triple locus variants.

ST	Frequency	SLV	DLV	TLV	Countries of origin
1	11	1	1	2	G, L, P, S
2	1	1	2	1	E
3*	1	-	-	-	SC
4	1	1	2	1	SC
5	3	2	2	0	G, E, P
6^F	4	3	1	0	E, L, SC

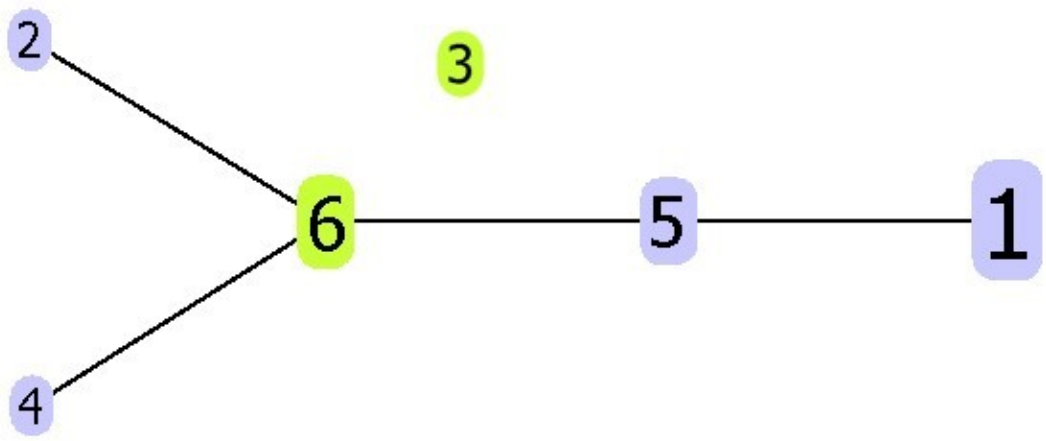


Fig. 6.4. goeBURST analysis of sequence types from 21 *Midichloria* samples, using 5 loci. ST 6 was predicted as founder population (shown in green), with STs 1, 2, 4 and 5 as derived strains (shown in light blue), and ST 3 as singleton. The size of the ST symbols corresponds to their relative frequency in the analysed population.

6.2.5 Phylogenetic comparison of *I. ricinus* ticks and their *Midichloria* endosymbionts

The concatenated gene sequences obtained from the mtMLST scheme established for *I. ricinus* (see chapter 3 of thesis) were used for a phylogenetic analysis of the 21 ticks that had been tested for their *Midichloria* endosymbiont sequences (see previous section). Their phylogenetic relationship was reconstructed by using the NJ method with 1,000 bootstrap replicates, as shown in Fig. 6.5. When compared to the NJ tree of their *Midichloria* endosymbionts (Fig. 6.3), both phylogenies displayed a common clustering of 11 taxa, of which 10 were identical in both trees. On the other hand, the tick phylogeny displayed a deeper structuring, and the formation of a sister clade containing three taxa (one each from England, Germany, and Switzerland, respectively) that was not mirrored in the *Midichloria* phylogeny. To allow an easier comparison of the two tree topologies both phylogenetic trees are displayed side by side in Fig. 6.6, with the tick phylogenetic tree on the left, and the *Midichloria* tree on the right. Eleven out of 21 taxa showed similar positions in both trees. The different scales of nucleotide differences in both trees should be noted.

An eBURST analysis of the tick STs is shown in Table 6.2. As already seen in the phylogenetic analysis in Fig. 6.5, the tick sequence data revealed a higher genetic diversity than their corresponding *Midichloria* endosymbionts. This is reflected in the high number of singletons (11) and the comparatively small proportion of samples grouped together in three different clonal complexes. These findings are visualised in Fig. 6.7.

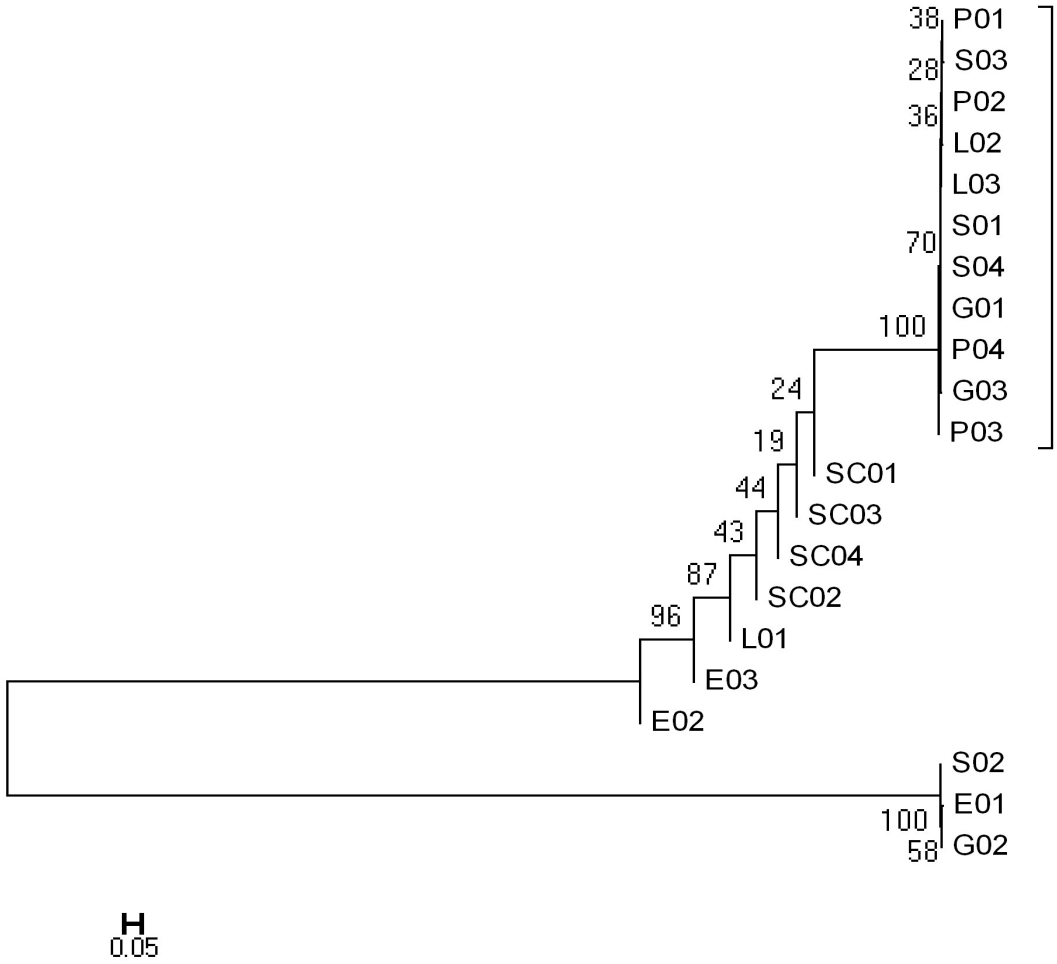


Fig. 6.5. Phylogenetic reconstruction inferred from the concatenated sequences of six gene fragments (12S, *atp6*, *cox1-3*, and *cytb*) of 21 *I. ricinus* ticks sampled in six different European countries using the Neighbour-Joining (NJ) method. The bootstrap consensus tree of 1000 repeats is shown. There were a total of 3198 positions in the final dataset. The scale bar represents 5% nucleotide difference. Square bracket indicates clustering of 11 samples similar to *Midichloria* phylogeny. Bootstrap values are shown on the branch nodes. E: England; G: Germany; L: Latvia; P: Portugal; S: Switzerland; SC: Scotland.

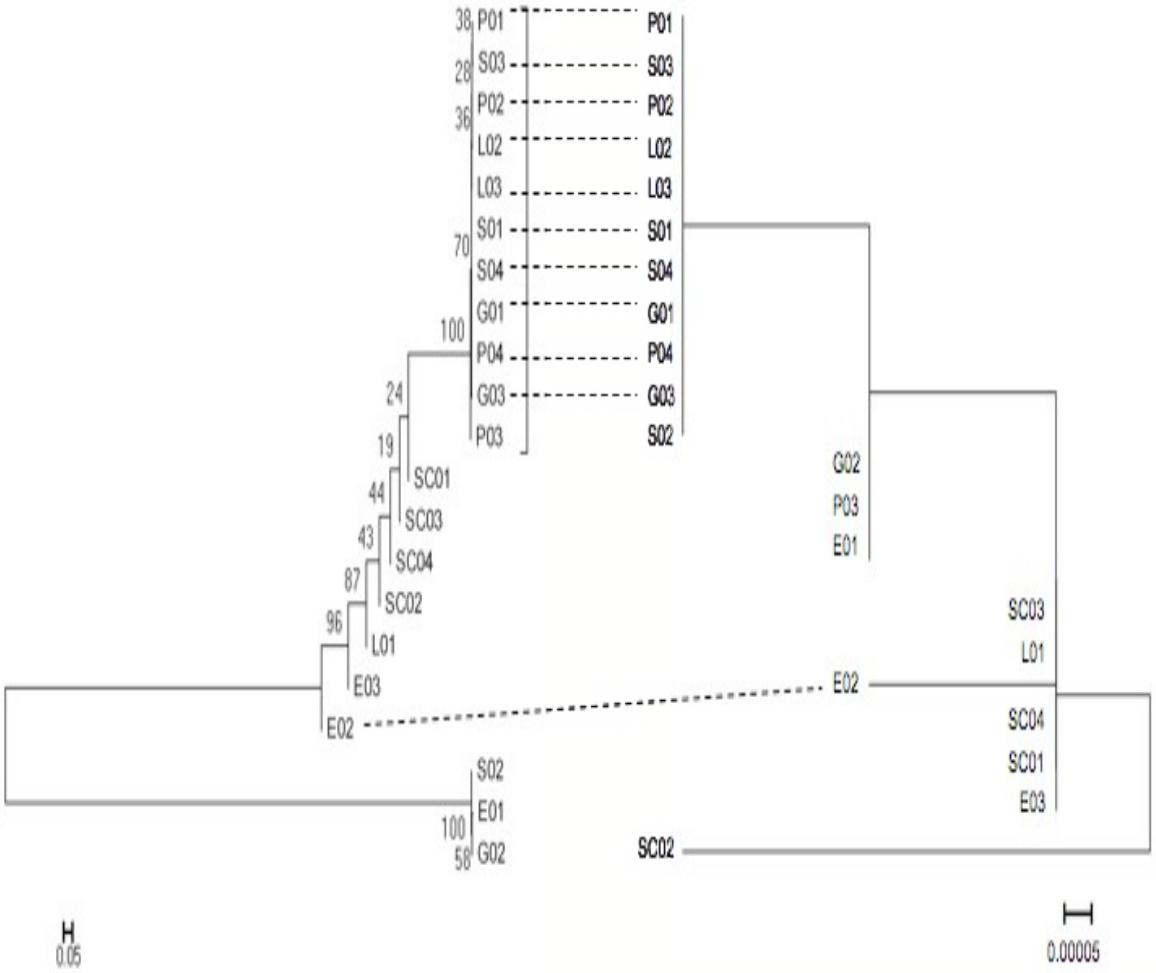


Fig. 6.6. Comparison of the phylogenetic reconstructions of 21 *I. ricinus* samples (left hand), and of their *Midichloria* endosymbionts (right hand). Dashed lines indicate similar positions in the respective trees. Bootstrap values are shown on the branch nodes. E: England; G: Germany; L: Latvia; P: Portugal; S: Switzerland; SC: Scotland.

Table 6.2. Statistics of eBURST analysis of 18 sequence types (STs) obtained from 21 *I. ricinus* sequences. Countries of origin for the tick samples are shown. E: England; G: Germany; L: Latvia; P: Portugal; S: Switzerland; SC: Scotland. ST 9 was predicted as founder strain (indicated by superscript F) of group 1. SLV: single locus variants; DLV: double locus variants; TLV: triple locus variants.

	ST	**Frequency**	**SLV**	**DLV**	**TLV**	**Countries**
Group 1	9^F	3	2	0	0	SC
	18	1	1	1	0	SC
	8	1	1	1	0	L
Group 2	4	2	1	0	0	G, S
	16	1	1	0	0	P
Group 3	3	1	1	0	0	S
	1	1	1	0	0	L
Singletons	17	1	-	-	-	S
	15	1	-	-	-	P
	14	1	-	-	-	E
	13	1	-	-	-	G
	12	1	-	-	-	P
	11	1	-	-	-	E
	10	1	-	-	-	E
	7	1	-	-	-	P
	6	1	-	-	-	L
	5	1	-	-	-	G
	2	1	-	-	-	S

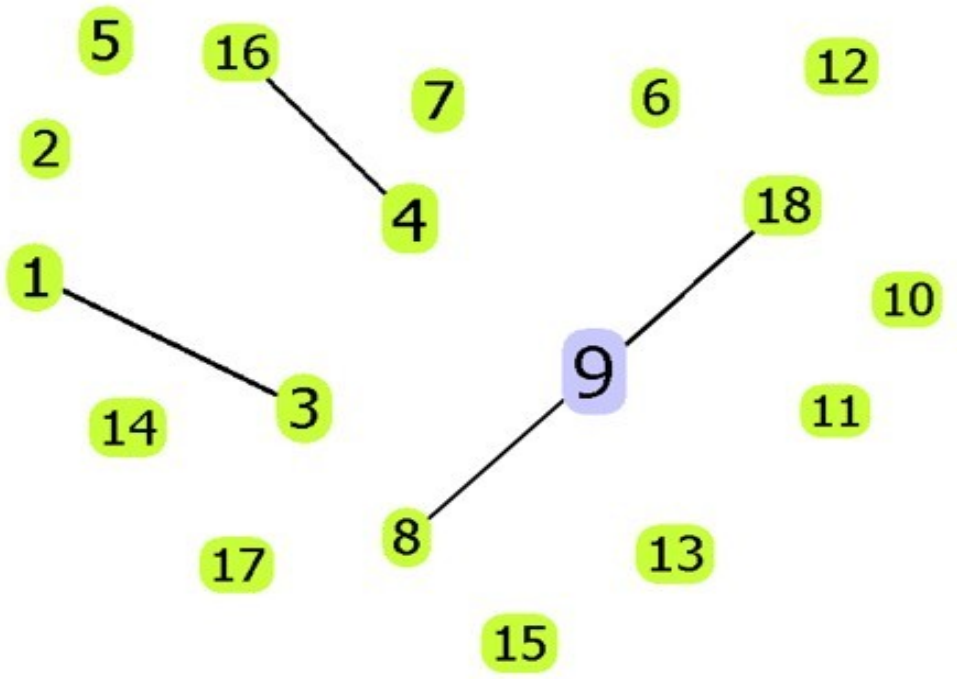


Fig. 6.7. goeBURST analysis of 18 sequence types (STs) from 21 *I. ricinus* samples, using 6 loci. ST 9 has been predicted as founder population (shown in light blue), with STs 8 and 18 as derived strains (shown in green), and with STs 1 and 3, and 4 and 16 as different groups. The size of the ST symbols corresponds to their relative frequency in the analysed population.

6.3 Discussion

6.3.1 Low prevalence of *Wolbachia* infections in British *I. ricinus* ticks

Due to its ability to influence the sex ratio of arthropod host populations, and because of its reported inhibition of infection with various pathogens in insect vectors (see section 6.1.1), *Wolbachia* has received significant attention as a potential mean to control vector populations. Recent studies revealed that infections with certain *Wolbachia* strains could block the transmission of the Dengue virus in the mosquito *Aedes aegypti* (Hoffmann et al., 2011; Walker et al., 2011). The control of tsetse flies (*Glossina* spp.), the vectors of *Trypanosoma brucei* parasites, which are the causative agent of sleeping sickness in humans, has also been suggested to focus on the mutualist endosymbiont *Wigglesworthia glossinidia* of these insects (reviewed by Aksoy et al., 2001). Apart from arthropod disease vectors, *Wolbachia* constitute obligate endosymbionts required for host fertility in certain nematodes and have successfully been targeted in river blindness-causing filarial nematodes (*Onchocerca volvulus*) (André et al., 2002; Hoerauf et al., 2000; 2001).

In this study, a *Wolbachia* prevalence of 10.8% (19 out of 176 tested ticks) was found in

I. ricinus ticks from the UK. To our knowledge this is the first screening of British ticks on a large scale. Previous studies found various rates of *Wolbachia* prevalence in *I. ricinus*, ranging from 0.2% (1 in 418 ticks) in ticks from Tunisia and Morocco (Sarih et al., 2005), and 0.9% in samples from Southern Germany (Hartelt et al., 2004), to approximately 5% in The Netherlands (F. Gassner, pers. comm.). *Wolbachia* positive signals were also detected in one out of seven *I. scapularis* ticks from the Northeastern United States (Benson et al., 2004). The prevalence rates observed in this study were significantly higher than in any of the previous studies on *I. ricinus* (for example a Fisher's exact test showed a value of $P < 0.0001$ when compared with the data from Sarih et al.). Potential reasons for these significant differences between samples from different countries could include climatic factors that might contribute to the spread of *Wolbachia* in a population. Another possibility would be that the prevalence of *Wolbachia* is in a non-equilibrium state, and that the British *I. ricinus* population is currently either ahead of European populations in a spreading *Wolbachia* infection, or lagging behind in a decline of infection rates. It would also be interesting to test whether a correlation exists between infections with borrelia and *Wolbachia*. However, given the low prevalence of borrelia of about 5% observed in British ticks (Vollmer et al., 2011), the expected frequency of a double infection of the two microorganisms would be quite low. Therefore a very large set of samples would have to be screened for both organisms with reliable and specific primers, which exceeded the limitations of my project in terms of time and money.

While the identity of amplified *wsp* sequences could be confirmed by a BLAST comparison for some of the samples that displayed strong gel bands, some weaker bands on the gel images (see Figs. 6.1, 6.2) could be the result of multiple binding sites for the primers within the target DNA. One difficulty in detecting *Wolbachia* in Acari by PCR could be that all published primer sequences for genes such as 16S, *wsp* and *ftsZ* have so far been designed for *Wolbachia* strains in insects (Braig et al, 1998; Casiraghi et al., 2005; O'Neill et al., 1992), which should stimulate further studies to obtain better information and design more specific primers for *Wolbachia* in ticks. Another possible explanation for the variation in band clarity could be if *Wolbachia* does not infect all tissue types of a tick with the same intensity. As our PCR tested total tick extracts this might lead to a mixed quality of amplifications as the PCR might be inhibited by too much DNA.

A statistical meta-analysis by Hilgenboecker and colleagues (2008) of infection rates

with *Wolbachia* in different arthropod species estimated that about 66% of all species were infected with *Wolbachia*, a higher rate than most previous studies had found. However, since most studies were focused on insects, this analysis remained vague about the number of non-insect species being infected. In this study, the authors also described a mathematical model which predicted that *Wolbachia* prevalence within one species would follow a 'most-or-few' pattern, with either many (> 90%) or few (< 10%) individuals within one species being infected (Hilgenboecker et al., 2008). Unlike many insect species, where *Wolbachia* prevalences can be very high, infections in ticks appear to fall into the latter group, which could limit the potential of using *Wolbachia* as a means of vector control in ticks.

6.3.2 Absence of *Cardinium* in British tick samples

Cardinium is a member of the Cytophaga-Flavobacterium-Bacteroides (CFB) phylum with an endosymbiont lifestyle and was first described in false spider mites (*Brevipalpus phoenicis*) by Weeks et al. (2001) and in parasitoid *Encarsia* wasps by Zchori-Fein et al. (2001). It was formally characterized as '*Candidatus* Cardinium hertigii' (Zchori-Fein et al., 2004) and is hereafter referred to as *Cardinium*. After *Wolbachia* (see previous section), it was only the second bacterial endosymbiont found to induce CI in arthropods, such as wasps (Hunter et al., 2003) and mites (Gotoh et al., 2007). Unlike *Wolbachia*, however, it seems to increase female rather than male mortality, thereby resulting in a reverse outcome on the sex bias in hosts (Ros & Breeuwer, 2009). Since then it has been found in various groups of arthropods, including mites (Enigl & Schausberger, 2007), and often in combination with *Wolbachia* (Duron et al., 2008; Ros & Breeuwer, 2009; Weeks et al., 2003; Zchori-Fein & Perlman, 2004).

Given the possible impact of this sex-distorting endosymbiont on populations of *I. ricinus*, similar to those described above for *Wolbachia*, it was decided to screen the same set of ticks for the presence of *Cardinium*.

The same set of ticks samples tested for *Wolbachia* (see section 6.2.1) was also tested for *Cardinium* by screening them for a fragment of the 16S rDNA (by using the primers CLOf and CLOr1, designed after Weeks et al., 2003), and by screening for gyrB (after Zchori-Fein & Perlman, 2004). No positive signals of amplified *Cardinium* sequences were observed in any of the analysed ticks (data not shown).

Cardinium has been detected in about 6-7% of all tested arthropod species, and appears to infect chelicerates more frequently than insects (Ros & Breeuwer, 2009). Within the Acari, however, detection of *Cardinium* has largely been confined to mites, but, similar to the results of this study, it was absent in several species of ixodid ticks (*Rhipicephalus* spp., *Boophilus annulatus*, *Hyalomma excavatum*; see Zchori-Fein & Perlman, 2004; *I. ricinus*, *I. uriae*; see Duron et al., 2008). Benson and colleagues (2004) on the other hand did find a positive signal for *Cardinium* in one out of seven *I. scapularis* ticks from the Northeastern US. Given these conflicting findings, a more exhaustive and thorough screening of larger numbers of individual ticks from different species and locations might reveal the true extent of *Cardinium* infections in ticks. As in the case of *Wolbachia*, a tissue-specific DNA approach may give better results.

6.3.3 Towards an MLST scheme for *Midichloria* endosymbionts in *I. ricinus*

Midichloria is a recently discovered endosymbiont in *I. ricinus* that is unique in its lifestyle of invading and multiplying inside mitochondria of tick cells. It has mainly been found in cells in the ovary tissue of female *I. ricinus*, from where it is transmitted to the tick's offspring, but it also occurs in male ticks, albeit at a lower prevalence (Lo et al., 2006). Our findings confirm the uniformly high prevalence of *Midichloria* in female tick samples, but also the lower prevalence in male ticks from various European countries. These findings could relate to the maternal inheritance of mitochondria and the vertical transmission of most *Midichloria* through the maternal line.

This study is the first attempt to establish an MLST scheme for *Midichloria* in *I. ricinus* samples from Europe, compared to previous studies that used only one or two loci (Epis et al., 2008; Lo et al., 2006). A phylogenetic analysis of a concatenated sequence of five housekeeping gene fragments revealed low levels of diversity and the clustering of 10 samples from mainland Europe, thereby demonstrating a small degree of geographical structuring (see Fig. 6.3). An eBURST analysis of the six sequence types (STs) found in the 21 samples showed high levels of clonality, with one ST (ST 1) comprising 11 of the samples, and all other STs containing only between one and four samples each (see Table 6.1). All except one sample (identified as ST 3) were clustered together in one clonal complex, with ST 6 as the predicted founder (see Fig. 6.4 for a goeBURST analysis). This high degree of clonality resembles those observed in an MLST-based study on *Bartonella quintana*, another endobacterial species of the order Rickettsiales (Arvand et al., 2010).

Here, the authors also found low levels of genetic difference between samples collected from different countries and in different years, and described the formation of only two clonal complexes of low diversity within the studied population.

The findings of *Midichloria* observed in the work for this chapter differ to some extent from the analysis of the corresponding tick samples through the mtMSLT scheme set up by our group. The phylogenetic reconstruction showed a clustering of 10 samples from mainland Europe in one clade (see Fig. 6.5) similar to that observed in the *Midichloria* tree (see Fig. 6.6 for a direct comparison of the two phylogenies), while the tick phylogeny had higher levels of nucleotide differences by about one order of magnitude. All other samples clustered in one medium sized and one smaller clade on both trees. To be conclusive, more phylogenetic information from rooted trees with stronger bootstrap support would be required. In an eBURST analysis the tick samples exhibited a high degree of genetic diversity, with the entire group of 21 samples consisting of 18 different STs, most of which (11) were singletons, while the remaining seven STs formed three small clonal complexes (see Table 6.2 and Fig. 6.7).

This partial incongruence of the phylogenies of *Midichloria* samples and their tick hosts confirms on a finer scale previous and within *I. ricinus* findings by Epis et al. (2008), who used smaller fragments of 16S rDNA of *Midichloria* and 12S mt rDNA from various tick species for a phylogenetic comparison. These observations raise the question whether other routes of transmission apart from the established vertical transmission route (from mother to offspring, see Beninati et al., 2004; Lo et al., 2006), such as through shared blood meal hosts, could also provide for a faster dissemination of *Midichloria* through the entire tick population (Epis et al., 2008).

With regard to the potential role of *Midichloria* in vector control of *I. ricinus*, these observations differ from previous findings by Chen et al. (1999), who described concordant phylogenies of tsetse flies (*Glossina* spp.) and their mutualistic endosymbiont *Wigglesworthia glossinidia*. It remains unclear whether infection with *Midichloria* has any beneficiary or negative effects on *I. ricinus*, but it has been suggested that *Midichloria* is a facultative mutualist, since it has been found in 100% of female ticks in the wild, but it can be lost by ticks reared in laboratory colonies without any apparent adverse outcomes (Sassera et al., 2011). This recent study by collaborators of our group included an analysis of the complete genome of *Midichloria* and aims to provide insights into the evolution of

mitochondria and their shared ancestry with *Rickettsia*-like α-Proteobacteria such as *Midichloria*. The establishment of an MLST scheme for *Midichloria* should improve the knowledge of evolutionary processes that form its population.

6.4 Conclusions

This chapter attempted to assess the prevalence of several bacterial endosymbionts in *I. ricinus* ticks from Britain and mainland Europe. Positive signals for *Wolbachia* sp. were found in 10.8% of British ticks, which is significantly higher than rates found in previous studies from mainland Europe. Signals for *Cardinium* sp. were absent in all of the screened samples. *Midichloria* sp., a recently discovered endosymbiont of tick mitochondria, was observed in 100% of all female ticks tested by PCR. A novel MLST scheme for this organism was established by amplifying five housekeeping gene fragments. The subsequent phylogenetic analysis revealed low levels of genetic diversity among samples from various European countries and substantial congruence with the phylogeny of the tick hosts. The high prevalence rate and degree of clonality in the *Midichloria* sp. samples suggest rapid horizontal transfer of these parasites across the tick population in addition to previously known vertical transmission.

7. Overall discussion and summary

7.1 Summary of findings

- **Chapter 3:** With my aid a novel mtMLST scheme for *I. ricinus* was established in our group. It could be shown that ticks from two sites near Bath, UK, did not differ in their phylogeographic signals. This is most likely the result of genetic exchange between the two sites facilitated by the movement of host animals such as deer. Interestingly, a clustering of borrelia-infected ticks from both sites could be observed.

  An analysis of ticks from Britain and Latvia revealed a clear geographic structuring in their phylogenetic reconstruction, indicating that postglacial isolation of the British Isles has prevented the regular exchange of genetic material between these separate tick populations. These findings differ from previous studies that did not observe genetic structuring within the European population of *I. ricinus*.

  An attempt to apply this novel mtMLST scheme to populations of *I. scapularis* in North America delivered only poor amplification results for ticks from the Southern clade, probably due to molecular incompatibility of these ticks with the originally developed primers. Those sequences that could be obtained from ticks from the Northeastern and Midwestern clades showed weak phylogeographic clustering, indicating again the existence of genetic exchange through hosts.
- **Chapter 4:** The ecological conditions of a woodland habitat and an ecotonal habitat in Southwestern England were compared in terms of host species composition, abundance, and tick density. Further biotic and abiotic factors included soil type, vegetation cover and plant communities, and climatic data. Tick densities were found to be higher in the woodland habitat, while the ecotonal site harboured higher densities of rodent hosts. While tick infestation rates on birds from a nearby site were similar to previous observations, tick infestation rates on rodents remained much lower than in comparable studies from mainland Europe, which supports earlier findings of low prevalence rates of rodent-associated borrelia spirochaetes in these sites. No borrelia were detected in ticks collected from rodents in this work.
- **Chapter 5:** A previously developed technique to analyse the host origins of tick blood meals was modified and adapted to the British fauna. Significant differences

in host utilisation by ticks from two different habitats was observed. It could be shown that repeated feeding of ticks, as shown by mixed host group signals, occurs more often than previously assumed. No ticks were found to feed on bank voles. Borrelia-infected ticks showed higher rates of positive signals than uninfected ticks, although the sample size of only 37 borrelia-positive ticks was quite low. Ticks collected in Britain also displayed higher positive rates than ticks from Latvia, which could be the result of DNA deterioration in these older samples, or because they had been feeding on hosts that were not detectable by the analysis used.

- **Chapter 6:** The endobacterial fauna of British *I. ricinus* ticks was analysed. *Wolbachia* sp., an ubitiquous endosymbiont known to affect the reproductive biology of other arthropod groups, in some cases affecting their sex ratio, was observed to be present in ca. 10% of all tested British samples. This rate is higher than what has been observed in previous European studies, but is similar to infection rates observed in *I. scapularis*. *Cardinium* sp., another endosymbiont that influences the sex ratio of its hosts, was absent in these samples.
  A recently discovered endosymbiont of tick mitochondria, *Midichloria mitochondrii*, was found in all female tick samples tested, and in most male samples. A multigene analysis of five housekeeping gene fragments showed a partially coordinated phylogeography of *M. mitochondrii* samples and their tick hosts from six different European countries.

7.2 Discussion

I. ricinus is the most important vector for Lyme borreliosis (LB) spirochaetes of the *B. burgdorferi s.l.* species complex in Europe (Gray, 2002). Hubalek (2009) estimated a total of about 65,000 annual cases of human LB in Europe, with an increasing trend. Yearly rates of infections seem to increase from Northern to Southern Europe, with average annual incidences per 100,000 cases ranging from 0.6 in Ireland, 0.7 in the UK, and 2.8 in Norway to 155 in Slovenia (EUCALB, 2011). Similarly, huge variations were observed in the prevalence of borrelia in questing ticks from different European countries (e.g. Gassner et al., 2008; Kampen et al., 2004; Vollmer et al., 2011). Therefore the study of the interactions between the tick vector, its vertebrate hosts and its bacterial fauna are crucial to improve our understanding of ecological processes that shape the epidemiology of LB and other tick-borne diseases. The findings made in this work should help to assess present

risks of these diseases, and to predict future developments in space and time.

The phylogeographic differentiation between British and Latvian *I. ricinus* ticks shown in Chapter 3 demonstrates how geographical separation can lead to phylogenetically distinct populations of this vector. This could have implications for the epidemiology of diseases such as LB. The coevolution of a pathogen such as borrelia and its host or vector can lead to increased fitness of the pathogen by specialization and a more efficient infection of the host, which in turn would result in the host evolving further to improve its fitness. This "arms race" between a parasite and its host is part of what has been coined "Red Queen theory" (van Valen, 1973). One interpretation of this theory by Gandon et al. (2008) predicts that in a tightly coevolved host/pathogen system, one would expect the infectiveness of the parasite to increase in the short term when tested against a previous generation of the host organism. Similarly, one would expect the ability of the host to resist infection when tested against a standard parasite genotype, representing a previous generation in the lineage of the parasite. However, it would be extremely difficult to test the hypothesis that antagonistic coevolution occurs between LB spirochaetes and their tick hosts. To do so, one would need to obtain various kinds of information, such as measurements of infectivity over a long period of time, information about generation time and the number of progeny, for both borrelia and ticks. This would require the ability to store both ticks and bacteria of standard genotypes over several generations, and to experimentally test the infection ability.

A study by Foley et al. (2008) compared the evolutionary history of *Ixodes* spp. ticks from Europe and North America and of *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis (HGA). Their findings were based on single-gene analyses and could not show clear signals of coevolution between pathogen and tick vector. To observe signals of antagonistic coevolution, the selection of loci that are affected by this selection pressure would be important. The housekeeping genes that are used in the respective MLST schemes for borrelia and for *I. ricinus* developed in our group, however, were chosen because they undergo neutral selection. This would make them less suitable to monitor changes in the fitness of the bacteria or the hosts than other loci that are non-neutrally selected, such as genes encoding outer surface proteins (Osp) in borrelia. An applied aspect of this research could be the development of potential vaccines against pathogens like borrelia, which would aim to avoid targeting rapidly evolving genes, such

as those affected by antagonistic coevolution.

An MLST-based comparison between bacteria and their tick hosts was made in Chapter 6 of this work, which illustrated the parallel phylogeography of *I. ricinus* and its endosymbiont *Midichloria sp.*, most likely as a result of horizontal transmission. It would be difficult to establish, however, how an infection with *Midichloria* affects the fitness of the tick host, as mentioned above for borrelia. The observed differences in *Midichloria* spp. infection rates between male and female *I. ricinus* resemble those observed previously for infections with borrelia (de Meeus et al., 2004). The possible interactions of infections with borrelia and other bacteria, such as *Wolbachia* or *Midichloria* spp., should be further investigated. Other findings made in this section include the high prevalence rate of *Wolbachia sp.* in British *I. ricinus* samples, and the absence of *Cardinium sp.*. As previously described, these two endobacterial species are capable of affecting the sex ratio in their arthropod hosts, which could have an impact on the composition of the host population. The possibility of using these bacteria as means of vector control in ticks deserves to be researched in future work.

The findings made in Chapter 4 compared for the first time different habitats in the Southwest of England and how ecological parameters in these habitats affect tick densities and host associations.

In Chapter 5, a blood meal analysis was carried out for the first time with *I. ricinus* ticks from different sites in Southwest England. These findings were compared with those from Latvian samples. It could also be shown that repeated feeding on different hosts occurs quite frequently, thus supporting previous studies (e.g Moran Cadenas et al., 2007). Interestingly, many British *I. ricinus* had fed as immature ticks on artiodactyl hosts such as deer, which would help to explain the low prevalence rates of borrelia found in ticks from these areas.

Given the observed differences in phylogenetic signals, in host association, and in endobacterial composition and abundance, one could speculate whether the *I. ricinus* population on the British Isles has evolved into a distinct subspecies, differing both genotypically and phenotypically from their counterparts in mainland Europe. Various characteristics of ticks have been suggested in a review by Magalhaes et al. (2007) to contribute to potential formations of subspecies (or "host races") in Acari, including

philopatry, low dispersal ability, a high intimacy with the host environment, host-associated mating, and reproductive rates. Several of these factors, such as philopatry, low dispersal rate, and a high intimacy with the host during the feeding process, are fulfilled by *I. ricinus* ticks.

Taken together, the results of this thesis combine:

- An analysis of the molecular ecology of *I. ricinus* and *I. scapularis* ticks, two important vector species of LB and other diseases
- The ecological context in which these vectors act
- The host associations of *I. ricinus*.
- The endobacterial fauna of *I. ricinus*.

This integrated approach of molecular and organism-based analyses is crucial in understanding the complex interactions of these vectors, their environment and their bacterial fauna, with further implications for the epidemiology of human diseases.

7.3 Future work

In order to improve our knowledge of the molecular evolution of *Ixodes* ticks, future work should include the development of a full mtMLST scheme for populations of *I. scapularis* in North America. The results of this novel scheme could be compared to earlier findings and could complement a currently developed analysis based on microsatellites (J. Tsao, pers. comm.). An mtMLST scheme would also allow to analyse the geographic distribution of different sequence types, and to compare these data with phylogeographic analyses of tick-borne pathogens, such as LB spirochaetes.

Given the differences in host associations between the different clades of *I. scapularis*, the application of a blood meal analysis similar to that carried out for *I. ricinus* in this study could deliver important results. A previous BMA study (Allan et al., 2010) tried to identify reservoir hosts for *Amblyomma americanum* ticks, and could provide initial information for the development of RLB oligo probes that are specific for the North

American fauna.

As mentioned above, a large-scale analysis of multiple infections with borrelia and *Wolbachia* sp. and/or *Midichloria* sp. would gain insight into the interactions of these bacterial parasites. The multi gene analysis of *Midichloria* sp. that was applied in this study should be expanded to a full MLST scheme by including more loci. This would increase the range of potential genetic variation that could observed in this scheme. It should also include more samples from different geographic sites to monitor the possible coevolution of the endosymbiont and its tick host.

7.4 Final summary

This study helped to establish a novel mtMLST scheme for the tick *I. ricinus*, an important ectoparasite and vector of various infectious diseases, including Lyme borreliosis. *I. ricinus* ticks from the Southwest of England formed a distinct clade when compared to ticks from Latvia, but showed no geographic structuring between two English sites with different ecologies. The two habitats in these sites differ in terms of vegetation and vertebrate host species composition and abundance. Both sites show low densities of rodents, and very few *I. ricinus* ticks infesting these hosts, while bird infestation rates in a nearby site are higher.

A blood meal analysis identified mammals, especially artiodactyl species, as the main hosts parasitised by immature ticks. Repeated feeding on different hosts was repeatedly observed. *I. ricinus* ticks from Britain showed higher rates of positive signals than ticks from Latvia, and borrelia-infected ticks showed higher rates of positive signals than uninfected ticks.

Higher rates of endobacterial *Wolbachia* sp. parasites were found in British *I. ricinus* samples than observed in earlier studies from other European countries. The unique bacterial endosymbiont *M. mitochondrii* was found in all female and most male tick samples tested. In a novel multi gene analysis it also showed signs of partial coevolution with its tick host.

8. Bibliography

Aksoy, S., Maudlin, I., Dale, C., Robinson, A. S., & Neill, S. L. O. (2001). Prospects for control of African trypanosomiasis by tsetse vector manipulation. *Trends Parasitol*, *17*, 29-35.

Allan, B. F., Goessling, L. S., Storch, G. A., & Thach, R. E. (2010). Blood meal analysis to identify reservoir hosts for *Amblyomma americanum* ticks. *Emerg Infect Dis*, *16*, 433-440.

Andersson, S. G., Karlberg, O., Canback, B., & Kurland, C. G. (2003). On the origin of mitochondria: a genomics perspective. *Philos Trans R Soc Lond B Biol Sci*, *358*, 165-169.

Andersson, S. G., Zomorodipour, A., Andersson, J. O., Sicheritz-Ponten, T., Alsmark, U. C., Podowski, R. M., et al. (1998). The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature*, *396*, 133-140.

André, A. S., Blackwell, N. M., Hall, L. R., Hoerauf, A., Brattig, N. W., Volkmann, L., et al. (2002). The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. *Science*, *295*, 1892-1895.

Apperson, C. S., Hassan, H. K., Harrison, B. A., Savage, H. M., Aspen, S. E., Farajollahi, A. R. Y., et al. (2004). Host feeding patterns of established and potential mosquito vectors of West Nile Virus in the Eastern United States. *Vector Borne Zoonotic Dis*, *4*, 71-82.

Apperson, C. S., Levine, J. F., Evans, T. L., Braswell, A., & Heller, J. (1993). Relative utilization of reptiles and rodents as hosts by immature *Ixodes scapularis* (Acari: Ixodidae) in the coastal plain of North Carolina, USA. *Exp Appl Acarol*, *17*, 719-731.

Archie, E. A., & Ezenwa, V. O. (2011). Population genetic structure and history of a generalist parasite infecting multiple sympatric host species. *Int J Parasitol*, *41*, 89-98.

Arthur, D. R. (1965). Ticks in Egypt in 1500 B.C.? *Nature*, *206*, 1060-1061.

Arvand, M., Raoult, D., & Feil, Edward J. (2010). Multi-locus sequence typing of a geographically and temporally diverse sample of the highly clonal human pathogen *Bartonella quintana*. *PloS one*, *5*, e9765.

Assadian, O., & Stanek, G. (2002). Theobald Smith - The discoverer of ticks as vectors of disease. *Wien Klin Wochenschr*, *114*, 479-481.

Avise, J. C., & Walker, D. (1998). Pleistocene phylogeographic effects on avian populations and the speciation process. *Proc Biol Sci*, *265*, 457-463.

Baldo, L., & Werren, J. H. (2007). Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. *Curr Microbiol*, *55*, 81-87.

Ballard, J. W. O., & Whitlock, M. C. (2004). The incomplete natural history of mitochondria. *Mol Ecol*, *13*, 729-744.

Barker, S.C., & Murrell, A. (2004). Systematics and evolution of ticks with a list of valid genus and species names. *Parasitology*, *129 Suppl*, S15-36.

Basáñez, M.-G., Razali, K., Renz, A., & Kelly, D. (2007). Density-dependent host choice by disease vectors: epidemiological implications of the ideal free distribution. *Trans R Soc Trop Med Hyg*, *101*, 256-269.

Beati, L., & Keirans, J. E. (2001). Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial *12S* ribosomal DNA gene sequences and morphological characters. *J Parasitol*, *87*, 32-48.

Beninati, T, Lo, N, Sacchi, L, Genchi, C., Noda, H., & Bandi, C. (2004). A novel alpha-Proteobacterium resides in the mitochondria of ovarian cells of the tick *Ixodes ricinus*. *Appl Environ Microbiol*, *70*, 2596-2602.

Beninati, T., Riegler, M., Vilcins, I.-M. E., Sacchi, L., McFadyen, R., Krockenberger, M., et al. (2009). Absence of the symbiont *Candidatus* Midichloria mitochondrii in the mitochondria of the tick *Ixodes holocyclu*s. *FEMS Microbiol Lett*, *299*, 241-247.

Benson, M. J., Gawronski, J. D., Eveleigh, D. E., & Benson, D. R. (2004). Intracellular symbionts and other bacteria associated with deer ticks (*Ixodes scapularis*) from Nantucket and Wellfleet, Cape Cod, Massachusetts. *Appl Environ Microbiol*, *70*, 616-620.

Bian, G., Xu, Y., Lu, P., Xie, Y., & Xi, Z. (2010). The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS pathogens*, *6*, e1000833.

Birky, C. (2001). The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Ann Rev Gen*, *35*, 125-148.

Black IV, W. C., & Piesman, J.. (1994). Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proc Natl Acad Sci U S A*, *91*, 10034-10038.

Black IV., W. C., Klompen, J. S., & Keirans, J. E. (1997). Phylogenetic relationships among tick subfamilies (Ixodida: Ixodidae: Argasidae) based on the 18S nuclear rDNA gene. *Mol Phylogenet Evol*, *7*, 129-144.

Boyard, C., Vourc'h, G. & Barnouin, J. 2008. The relationships between *Ixodes ricinus* and small mammal species at the woodland-pasture interface. *Exp Appl Acarol*, 44, 61-76.

Braig, H., Zhou, W., Dobson, S., & O'Neill, S. (1998). Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *J Bacteriol*, *180*, 2373-2378.

Brinkerhoff, R. J., Bent, S. J., Folsom-O'Keefe, C. M., Tsao, K., Hoen, A. G., Barbour, A. G., et al. (2010). Genotypic diversity of *Borrelia burgdorferi* strains detected in *Ixodes scapularis* larvae collected from North American songbirds. *Appl Environ Microbiol*, *76*, 8265-8268.

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., & Davis, J. P. (1982). Lyme disease - a tick-borne spirochetosis? *Science*, *216*, 1317-1319.

Burlini, L., Teixeira, K., Szabo, M., & Famadas, K. (2010). Molecular dissimilarities of *Rhipicephalus sanguineus* (Acari: Ixodidae) in Brazil and its relation with samples throughout the world: is there a geographical pattern? *Exp App Acarol*, *50*, 361-374.

Caporale, D. A., Rich, S. M., Spielman, A., Telford III., S. R., & Kocher, T. D. (1995). Discriminating between *Ixodes* ticks by means of mitochondrial DNA sequences. *Mol Phylogenet Evol*, *4*, 361-365.

Casati, S., Bernasconi, M. V., Gern, L., & Piffaretti, J. C. (2008). Assessment of intraspecific mtDNA variability of European *Ixodes ricinus* sensu stricto (Acari: Ixodidae). *Infect Genet Evol*, *8*, 152-158.

Casiraghi, M., Bordenstein, S. R., Baldo, L., Lo, N, Beninati, T, Wernegreen, J. J., et al. (2005). Phylogeny of *Wolbachia pipientis* based on gltA, groEL and ftsZ gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. *Microbiology*, *151*, 4015-4022.

CDC. (2007). Lyme disease--United States, 2003-2005. *MMWR Morb Mortal Wkly Rep*, *56*, 573-576.

CDC. (2011). Lyme Disease data and statistics. [Webpage]. Retrieved August 24, 2011, from http://www.cdc.gov/lyme/stats/index.html

Chen, X., Li, S., & Aksoy, S. (1999). Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. *J Mol Evol*, *48*, 49-58.

Chmelar, J., Anderson, J. M., Mu, J., Jochim, R. C., Valenzuela, J. G., & Kopecky, J. (2008). Insight into the sialome of the castor bean tick, *Ixodes ricinus*. *BMC Genomics*, *9*, 233.

Churchfield, S. (2008). Chapter 7: Insectivores, orders Erinaceomorpha and Soricomorpha. *Mammals of the British Isles: Handbook, 4th Ed.* (pp. 241-291). Southampton: The Mammal Society.

Comstedt, P., Bergstrom, S., Olsen, B., Garpmo, U., Marjavaara, L., Mejlon, H., et al. (2006). Migratory passerine birds as reservoirs of Lyme borreliosis in Europe. *Emerg Infect Dis*, *12*, 1087-1095.

Craine, N. G., Nuttall, P. A., Marriott, A. C., & Randolph, S. E. (1997). Role of grey squirrels and pheasants in the transmission of *Borrelia burgdorferi* sensu lato, the Lyme disease spirochaete, in the U.K. *Folia Parasitol (Praha)*, *44*, 155-160.

Craine, N. G., Randolph, S. E., & Nuttall, P. A. (1995). Seasonal variation in the role of grey squirrels as hosts of *Ixodes ricinus*, the tick vector of the Lyme disease spirochaete, in a British woodland. *Folia Parasitol (Praha)*, *42*, 73-80.

Cywinska, A., Hunter, F. F., & Hebert, P. D. (2006). Identifying Canadian mosquito species through DNA barcodes. *Med Vet Entomol*, *20*, 413-424.

Daniel, M., Kolár, J., Zeman, P., Pavelka, K., Sádlo, J. 1998. Predictive map of *Ixodes ricinus* high-incidence habitats and a tick-borne encephalitis risk assessment using satellite data. *Exp Appl Acarol*, 22, 417-433.

de Boer, R., Hovius, K. E., Nohlmans, M. K., Gray, J. S. 1993. The woodmouse (*Apodemus sylvaticus*) as a reservoir of tick-transmitted spirochetes (*Borrelia burgdorferi*) in The

Netherlands. *Zentralbl Bakteriol*, 279, 404-416.

de Meeus, T., Beati, L., Delaye, C., Aeschlimann, A., & Renaud, F. (2002). Sex-biased genetic structure in the vector of Lyme disease, *Ixodes ricinus*. *Evolution*, *56*, 1802-1807.

de Meeus, T., Lorimier, Y., & Renaud, F. (2004). Lyme borreliosis agents and the genetics and sex of their vector, *Ixodes ricinus*. *Microbes Infect*, *6*, 299-304.

de Meeus, T., McCoy, K. D., Prugnolle, F., Chevillon, C., Durand, P., Hurtrez-Bousses, S., et al. (2007). Population genetics and molecular epidemiology or how to "debusquer la bete." *Infect Genet Evol*, *7*, 308-332.

Delaye, C., Beati, L., Aeschlimannj, A., Renaud, F., & de Meeus, T. (1997). Population genetic structure of *Ixodes ricinus* in Switzerland from allozymic data: no evidence of divergence between nearby sites. *Int J Parasitol*, *27*, 769-773.

Dennis, D. T., Nekomoto, T. S., Victor, J. C., Paul, W. S., & Piesman, J. (1998). Reported distribution of *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae) in the United States. *J Med Entomol*, *35*, 629-638.

Despommier, D., Ellis, B. R. & Wilcox, B. (2006). The Role of ecotones in emerging infectious diseases. *EcoHealth*, 3, 281-289.

Dinnis, R. E. (2010). *Analysing the genetic diversity of* Ixodes ricinus *ticks using multilocus sequence typing*. PhD thesis. University of Bath, UK.

Diuk-Wasser, M. A., Vourc'h, G., Cislo, P., Gatewood-Hoen, A., Melton, F., Hamer, S., et al. (2010). Field and climate-based model for predicting the density of host-seeking nymphal *Ixodes scapularis*, an important vector of tick-borne disease agents in the eastern United States. *Global Ecol Biogeogr*, *19*, 504-514.

Dizij, A., & Kurtenbach, K. (1995). *Clethrionomys glareolus*, but not *Apodemus flavicollis*, acquires resistance to *Ixodes ricinus* L., the main European vector of *Borrelia burgdorferi*. *Parasite Immunol*, *17*, 177-183.

Douady, C. J., Dosay, M., Shivji, M. S., & Stanhope, M. J. (2003). Molecular phylogenetic evidence refuting the hypothesis of Batoidea (rays and skates) as derived sharks. *Mol Phylogen Evol*, *26*, 215-221.

Dubska, L. Literak, I., Kocianova, E., Taragelova, V., Sychra, O. 2009. Differential role of passerine birds in distribution of *Borrelia* spirochetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe. *Appl Environ Microbiol*, *75*, 596-602.

Durden, L. A, Oliver, J. H. O. J., Banks, C. W., & Vogel, G. N. (2002). Parasitism of lizards by immature stages of the blacklegged tick, *Ixodes scapularis* (Acari, Ixodidae). *Exp Appl Acarol*, *26*, 257-266.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstadter, J., et al. (2008). The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biol*, *6*, 27.

Dyer, N. A., Ravel, S., Choi, K.-shik, Darby, A. C., Causse, S., Hall, M. J. R., et al. (2011). Cryptic diversity within the major trypanosomiasis vector *Glossina fuscipes* revealed by molecular markers. *PLoS Negl Trop Dis*, *5*, 1-12.

Eisen, L., Eisen, R.J. & Lane, R.S., 2004. The roles of birds, lizards, and rodents as hosts for the western black-legged tick *Ixodes pacificus*. *J Vector Ecol*, 29, 295-308.

Eisen, R., & Lane, L. (2002). Vectors of *Borrelia burgdorferi sensu lato.* In O. K. J. Gray R. S. Lane, and G. Stanek (Ed.), *Lyme Borreliosis Biology, Epidemiology and Control* (pp. 91-115). Wallingford, Oxon, United Kingdom: CAB International.

Emerson, B. C., & Hewitt, G. M. (2005). Phylogeography. *Curr Biol*, *15*, R367-371.

Enigl, M., & Schausberger, P. (2007). Incidence of the endosymbionts *Wolbachia*, *Cardinium* and *Spiroplasma* in phytoseiid mites and associated prey. *Exp Appl Acarol*, *42*, 75-85.

Enright, M. C., & Spratt, B. G. (1999). Multilocus sequence typing. *Trends Microbiol*, *7*, 482-487.

Enzensberger, U. (2001). *Parasiten*. Frankfurt a. M.: Eichborn Verlag.

Epis, S, Sassera, D, Beninati, T, Lo, N, Beati, L., Piesman, J., et al. (2008). *Midichloria mitochondrii* is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. *Parasitology*, *135*, 485-494.

Estrada-Pena, A., Daniel, M., Frandsen, F., Gern, L., Gettinby, G., Gray, J. S., et al. (1998). *Ixodes ricinus* strains in Europe. *Zentralbl Bakteriol*, *287*, 185-189.

Estrada-Peña, A., Osácar, J. J., Pichon, B., & Gray, J. S. (2005). Hosts and pathogen detection for immature stages of *Ixodes ricinus* (Acari: Ixodidae) in North-Central Spain. *Exp Appl Acarol*, *37*, 257-268.

Etti, S., Hails, R., Schafer, S. M., De Michelis, S., Sewell, H. S., Bormane, A, et al. (2003). Habitat-specific diversity of *Borrelia burgdorferi sensu lato* in Europe, exemplified by data from Latvia. *Appl Environ Microbiol*, *69*, 3008-3010.

EUCALB. (2011). Epidemiology of European Lyme Borreliosis. [Webpage]. Retrieved September 11, 2011, from http://meduni09.edis.at/eucalb/cms/index.php?option=com_content&task=view&id=118&Itemid=115

Faulde, M. K. & Robbins, R. G. (2008). Tick infestation risk and *Borrelia burgdorferi* s. l. infection-induced increase in host-finding efficacy of female *Ixodes ricinu*s under natural conditions. *Exp Appl Acarol*. *44*, 137-145.

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P., & Spratt, B. G. (2004). eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol*, *186*, 1518-1530.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*, 783.

Filippova, N. A. (1991). A hypothesis for the palaeogenesis of the distribution of the main vector

for Lyme disease. In *Modern acarology*. B. V. Dusbabek F (Ed.), (pp. 109-118). The Hague, Netherlands: SPB Academic Publishing.

Filippova, N. A. (2002). Forms of sympatry and possible ways of microevolution of closely related species of the group *Ixodes ricinus persulcatus* (Ixodidae). *Acta Zoologica Lituanica*, *12*, 215-227.

Fitch, S., Thomson, K., & Gaffney, V. (2005). Late pleistocene and holocene depositional systems and the palaeogeography of the Dogger bank, North Sea. *Quartern Res*, *64*, 185-196.

Foley, J., Nieto, N. C., Foley, P., & Teglas, M. B. (2008). Co-phylogenetic analysis of *Anaplasma phagocytophilum* and its vectors, *Ixodes spp.* ticks. *Exp Appl Acarol*, *45*, 155-170.

Francisco, A. P., Bugalho, M., Ramirez, M., & Carriço, J. A. (2009). Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. *BMC Bioinformatics*, *10*, 1-15.

Fuller, R., & Gill, R. (2001). Ecological impacts of increasing numbers of deer in British woodland. *Forestry*, *74*, 193-199.

Gandon, S., Buckling, A., Decaestecker, E., Day, T. (2008). Host-parasite coevolution and patterns of adaptation across time and space. *J Evol Biol, 21,* 1861-1866.

Gassner, F., Verbaarschot, P., Smallegange, R. C., Spitzen, J., Van Wieren, S. E., et al. (2008). Variations in *Ixodes ricinus* density and *Borrelia* infections associated with cattle introduced into a woodland in the Netherlands. *Appl Environ Microbiol*. *74*, 7138-7144.

Gassner, F. (2010). *Tick tactics. Interactions between habitat characteristics, hosts and microorganisms in relation to the biology of the sheep tick* Ixodes ricinus. PhD thesis, University of Wageningen, NL.

Gassner, F., van Vliet, A. J. H., Burgers, S. L. G. E., Jacobs, F., Verbaarschot, P., Hovius, E. K. E., et al. (2011). Geographic and temporal variations in population dynamics of *Ixodes ricinus* and associated *Borrelia* infections in The Netherlands. *Vector Borne Zoonotic Dis*, *11*, 523-532.

Gatewood, A. G., Liebman, K. A., Vourc'h, G., Bunikis, J., Hamer, S. A., Cortinas, R., et al. (2009). Climate and tick seasonality are predictors of *Borrelia burgdorferi* genotype distribution. *Appl Environ Microbiol*, *75*, 2476-2483.

Geraci, N. S., Spencer Johnston, J., Paul Robinson, J., Wikel, S. K., & Hill, C. A. (2007). Variation in genome size of argasid and ixodid ticks. *Insect Biochem Mol Biol*, *37*, 399-408.

Gern, L, & Humair, P. (2002). Ecology of *Borrelia burgdorferi sensu lato* in Europe. In J. Gray, O. Kahl, R. Lane, & G. Stanek (Eds.), *Lyme borreliosis: biology, epidemiology and control* (pp. 149-174). Wallingford, Oxon, United Kingdom: CAB International.

Gern, L, Estrada-Pena, A., Frandsen, F., Gray, J. S., Jaenson, T. G., Jongejan, F., et al. (1998). European reservoir hosts of *Borrelia burgdorferi sensu lato*. *Zentralbl Bakteriol*, *287*, 196-204.

Gern, L., & Rais, O. (1996). Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). *J Med Entomol*, *33*, 189-192.

Glaser, R. L., & Meola, M. A. (2010). The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile Virus infection. *PloS one*, *5,* e11977.

Gotoh, T., Noda, H., & Ito, S. (2007). *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity*, *98*, 13-20.

Gray, J. S. (1991). The development and seasonal activity of the tick *Ixodes ricinus*: a vector of Lyme borreliosis. *Rev Med Vet Entomol*, *79*, 323-333.

Gray, J. S. (2002). Biology of *Ixodes* species ticks in relation to tick-borne zoonoses. *Wien Klin Wochenschr*, *114*, 473-478.

Gray, J. S., Kahl, O., Janetzki, C., & Stein, J. (1992). Studies on the ecology of Lyme disease in a deer forest in County Galway, Ireland. *J Med Entomol*, *29*, 915-920.

Gray, J. S., Robertson, J. N., & Key, S. (2000). Limited role of rodents as reservoirs of *Borrelia burgdorferi* sensu lato in Ireland. *Eur J Epidemiol*, *16*, 101-103.

Gray, J. S., Kahl, O., Janetzki-Mittman, C., Stein, J., Guy, E. (1994). Acquisition of *Borrelia burgdorferi* by *Ixodes ricinus* ticks fed on the European hedgehog, *Erinaceus europaeus*, L. *Exp Appl Acarol*, 18, 485-491.

Gray, J.S., Kirstein, F., Robertson, J. N., Stein, J., Kahl, O. (1999). *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks and rodents in a recreational park in South-western Ireland. *Exp Appl Acarol*, 23, 717-729.

Gray, J.S. (1998). The ecology of ticks transmitting Lyme borreliosis. *Exp Appl Acarol*, 22, 249-258.

Gray, M. W., Burger, G., & Lang, B. F. (1999). Mitochondrial evolution. *Science*, *283*, 1476-1481.

Guglielmone, A. A., Robbins, R. G., Apanaskevich, D. A., Petney, T. N., Estrada-Peña, A., Horak, I. G., et al. (2010). The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. *Zootaxa*, *2528*, 1 - 28.

Gurnell, J., & Flowerdew, J. R. (2006). *Live trapping small mammals: A practical guide* (4th ed., p. 48). London, UK: The Mammal Society.

Gurnell, J., & Hare, E. J. (2008). Chapter 5: Rodents, order Rodentia. In S. Harris & D. W. Yalden (Eds.), *Mammals of the British Isles: Handbook, 4th Ed.* (4th ed., pp. 54-199). Southampton: The Mammal Society.

Halos, L., Bord, S., Cotté, V., Gasqui, P., Abrial, D., Barnouin, J., et al. (2010). Ecological factors characterizing the prevalence of bacterial tick-borne pathogens in *Ixodes ricinus* ticks in pastures and woodland. *Appl Environ Microbiol*, 76, 4413-4420.

Hamer, S. A, Tsao, J. I., Walker, E. D., & Hickling, G. J. (2010). Invasion of the Lyme disease

vector *Ixodes scapularis*: implications for *Borrelia burgdorferi* endemicity. *EcoHealth*, *7*, 47-63.

Hanincova, K., Kurtenbach, K, Diuk-Wasser, M, Brei, B., & Fish, D. (2006). Epidemic spread of Lyme borreliosis, Northeastern United States. *Emerg Infect Dis*, *12*, 604-611.

Hanincova, K., Schafer, S. M., Etti, S., Sewell, H. S., Taragelova, V., Ziak, D., et al. (2003a). Association of *Borrelia afzelii* with rodents in Europe. *Parasitology*, *126*, 11-20.

Hanincova, K., Taragelova, V., Koci, J., Schafer, S. M., Hails, R., Ullmann, A. J., et al. (2003b). Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. *Appl Environ Microbiol*, *69*, 2825-2830.

Haouas, N., Pesson, B., Boudabous, R., Dedet, J.-pierre, Babba, H., & Ravel, C. (2007). Development of a molecular tool for the identification of *Leishmania* reservoir hosts by blood meal analysis in the insect vectors. *Am J Trop Med Hyg*, *77*, 1054-1059.

Harrison, A., Newey, S., Gilbert, L., Haydon, D. T., & Thirgood, S. (2010). Culling wildlife hosts to control disease: mountain hares , red grouse and louping ill virus. *J Appl Ecol*, *47*, 926-930.

Hartelt, K., Oehme, R., Frank, H., Brockmann, S. O., Hassler, D., & Kimmig, P. (2004). Pathogens and symbionts in ticks: prevalence of *Anaplasma phagocytophilum* (*Ehrlichia sp.*), *Wolbachia sp.*, *Rickettsia sp.*, and *Babesia sp.* in Southern Germany. *Int J Med Microbiol*, *293 Suppl*, 86-92.

Hasegawa, M., Kishino, H., & Yano, T. (1985). Dating the Human-Ape splitting by a molecular clock of mitochondrial DNA. *Mol Evol*, *22*, 160-174.

Hewitt, G. M. (2004). Genetic consequences of climatic oscillations in the quaternary. *Philos Trans R Soc Lond B Biol Sci*, *359*(1442), 183-195.

Heylen, D.J., Madder, M. & Matthysen, E. (2010). Lack of resistance against the tick *Ixodes ricinus* in two related passerine bird species. *Int J Parasitol*, *40*, 183-191.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., & Werren, J. H. (2008). How many species are infected with *Wolbachia*?--A statistical analysis of current data. *FEMS Microbiol Lett*, *281*, 215-220.

Hill, C. A., & Wikel, S. K. (2005). The *Ixodes scapularis* Genome Project: an opportunity for advancing tick research. *Trends Parasitol*, *21*, 151-153.

Hillyard, P. D. (1996). *Ticks of North-West Europe.* (C. J. H. Barnes RSK, Ed.) (Vol. 52). London, UK: The Linnean Society of London.

Hoen, A. G., Margos, G., Bent, S. J., Diuk-Wasser, M. A, Barbour, A., Kurtenbach, K., et al. (2009). Phylogeography of *Borrelia burgdorferi* in the Eastern United States reflects multiple independent Lyme disease emergence events. *Proc Nat Acad Sci USA*, *106*, 15013-15018.

Hoerauf, A., Volkmann, L., Hamelmann, C., Adjei, O, Autenrieth, I., Fleischer, B, et al. (2000). Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. *Lancet*, *355*, 1242-1243.

Hoerauf, A., Mand, S., Adjei, O., Fleischer, B., & Büttner, D. W. (2001). Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilaridermia after ivermectin treatment. *The Lancet*, *357*, 1415-1416.

Hoffmann, A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F., et al. (2011). Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*, *476*, 454-457.

HPA. (2011). Epidemiology of Lyme borreliosis in the UK. [Webpage]. Retrieved September 7, 2011, from http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/LymeDisease/EpidemiologicalData/lymLymeepidemiology/

Hu, C. M., Cheminade, Y., Perret, J.-L., Weynants, V., Lobet, Y., & Gern, L.. (2003). Early detection of *Borrelia burgdorferi sensu lato* infection in Balb/c mice by co-feeding *Ixodes ricinus* ticks. *Int J Med Microbiol*, *293*, 421-426.

Hu, R., Hyland, K., & Markowski, D. (1997). Effects of *Babesia microti* infection on feeding pattern, engorged body weight, and molting rate of immature *Ixodes scapularis* (Acari: Ixodidae). *J Med Entomol*, *34*, 559-564.

Hubalek, Z. (2009). Epidemiology of Lyme Borreliosis. In D. Lipsker & B. Jaulhac (Eds.), *Lyme Borreliosis: Biological and Clinical Aspects* (pp. 31-50). Basel: Karger.

Hughes, G. L., Koga, R., Xue, P., Fukatsu, T., & Rasgon, J. L. (2011). *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS Pathogens*, *7*, 3-10.

Hughes, V. L., & Randolph, S. E. (2001b). Testosterone increases the transmission potential of tick-borne parasites. *Parasitology*, *123*, 365-371.

Hughes, V. L. & Randolph, S. E. (2001a). Testosterone depresses innate and acquired resistance to ticks in natural rodent hosts: a force for aggregated distributions of parasites. *J Parasitol*, *87*, 49-54.

Humair, P. F., Douet, V., Cadenas, F. M., Schouls, L. M., Van De Pol, I., & Gern, L. (2007). Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker. *J Med Entomol*, *44*, 869-880.

Humair, P. F., Turrian, N., Aeschlimann, A., Gern, L. (1993). *Ixodes ricinus* immatures on birds in a focus of Lyme borreliosis. *Folia Parasitol (Praha)*, *40*, 237-242.

Humair, P. F., Rais, O. & Gern, L. (1999). Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance. *Parasitology*, *118* , 33-42.

Humphrey, P. T., Caporale, D. A., & Brisson, D. (2010). Uncoordinated phylogeography of *Borrelia Burgdorferi* and its tick vector, *Ixodes Scapularis*. *Evolution*, *64*, 2653-2663.

Hunter, M. S., Perlman, S. J., & Kelly, S. E. (2003). A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proc*

Royal Soc B, *270*, 2185-2190.

Huson, D. H., Richter, D. C., Rausch, C., Dezulian, T., Franz, M., & Rupp, R. (2007). Dendroscope: an interactive viewer for large phylogenetic trees. *BMC Bioinformatics*, *8*, 460.

Jaenson, T. G., & Talleklint, L. (1992). Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete. *J Med Entomol*, *29*, 813-817.

Jeyaprakash, A., & Hoy, M. A. (2009). First divergence time estimate of spiders, scorpions, mites and ticks (subphylum: Chelicerata) inferred from mitochondrial phylogeny. *Exp Appl Acarol*. *47*, 1-18.

Jongejan, F., & Uilenberg, G. (2004). The global importance of ticks. *Parasitology*, *129 Suppl*, S3-14.

Kambris, Z., Blagborough, A. M., Pinto, S. B., Blagrove, M. S. C., Godfray, H. C. J., Sinden, R. E., et al. (2010). *Wolbachia* stimulates immune gene expression and inhibits *Plasmodium* development in *Anopheles gambiae*. *PLoS Pathogens*, *6*, e1001143.

Kampen, H., Rotzel, D. C., Kurtenbach, K, Maier, W. A., & Seitz, H. M. (2004). Substantial rise in the prevalence of Lyme borreliosis spirochetes in a region of western Germany over a 10-year period. *Appl Environ Microbiol*, *70*, 1576-1582.

Keirans, J., Needham, G., & Oliver Jr., J. H. (1999). The *Ixodes ricinus* complex worldwide: Diagnosis of the species in the complex, hosts and distribution. In G. Needham (Ed.), *Acarology IX: Symposia* (pp. 341-346).

Kent, R. J. (2009). Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. *Mol Ecol Res*, *9*, 4-18.

Kent, R. J., Thuma, P. E., Mharakurwa, S., & Norris, D. E. (2007). Seasonality, blood feeding behaviour, and transmission of *Plasmodium falciparum* by *Anopheles arabiensis* after an extended drought in Southern Zambia. *Am J Trop Med Hyg*, *76*, 267-274.

Kenward, R. E., Hodder, K., Rose, R., Walls, C., Parish, T., Holm, J., et al. (1998). Comparative demography of red squirrels (*Sciurus vulgaris*) and grey squirrels (*Sciurus carolinensis*) in deciduous and conifer woodland. *J Zool*, 244, 7-21.

Kenward, R. E. & Tonkin, J. M. (1986). Red and grey squirrels: some behavioural and biometric dfferences. *J Zool*, 209, 279-281.

Kikkawa, J. (1964). Movement, activity and distribution of the small rodents *Clethrionomys glareolus* and *Apodemus sylvaticus* in woodland. *J Animal Ecol*, *33*, 259-299.

Kilpatrick, A. M., Kramer, L. D., Jones, M. J., Marra, P. P., & Daszak, P. (2006). West Nile Virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS Biology*, *4*.

Kimura, K., Isogai, E., Isogai, H., Kamewaka, Y., Nishikawa, T., Ishii, N., et al. (1995). Detection of Lyme Disease spirochetes in the skin of naturally infected wild sika deer (*Cervus nippon yesoensis*) by PCR. *Appl Environ Microbiol*, *61*, 1641-1642.

Kirstein, F., & Gray, J. S. (1996). A molecular marker for the identification of the zoonotic reservoirs of Lyme borreliosis by analysis of the blood meal in its European vector *Ixodes ricinus*. *Appl Environ Microbiol*, *62*, 4060-4065.

Kjelland, V., Ytrehus, B., Stuen, S., Skarpaas, T., & Slettan, A. (2011). Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks collected from moose (*Alces alces*) and roe deer (*Capreolus capreolus*) in southern Norway. *Ticks Tick-borne Dis*, *2*, 99-103.

Kurtenbach, K., De Michelis, S., Etti, S., Schafer, S. M., Sewell, H. S., Brade, V., et al. (2002). Host association of *Borrelia burgdorferi sensu lato*--the key role of host complement. *Trends Microbiol*, *10*, 74-79.

Kurtenbach, K., De Michelis, S., Sewell, H. S., Etti, S., Schafer, S. M., Hails, R., et al. (2001). Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe. *Appl Environ Microbiol*, *67*, 4926-4929.

Kurtenbach, K., Hanincova, K., Tsao, J. I., Margos, G., Fish, D., & Ogden, N. H. (2006). Fundamental processes in the evolutionary ecology of Lyme borreliosis. *Nat Rev Microbiol*, *4*, 660-669.

Kurtenbach, K., Kampen, H., Dizij, A., Arndt, S., Seitz, H. M., Schaible, U. E., et al. (1995). Infestation of rodents with larval *Ixodes ricinus* (Acari: Ixodidae) is an important factor in the transmission cycle of *Borrelia burgdorferi* s. l. in German woodlands. *J Med Entomol*, *32*, 807-817.

Kurtenbach, K., Peacey, M., & Rijpkema, S. (1998a). Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. *Appl Environ Microbiol*, *64*, 1169-1174.

Kurtenbach, K., Sewell, H. S., Ogden, N. H., Randolph, S. E., & Nuttall, P. A. (1998b). Serum complement sensitivity as a key factor in Lyme disease ecology. *Infect Immun*, *66*, 1248-1251.

Kurtenbach, K., Carey, D., Hoodless, A. N., Nuttall, P. A., & Randolph, S. E. (1998c). Competence of pheasants as reservoirs for Lyme disease spirochetes. *J Med Entomol*, *35*, 77-81.

Lane, R. S., Mun, J., Peribáñez, M. A., Stubbs, H. A., Lane, R. S., Mun, J., et al. (2007). Host-seeking behavior of *Ixodes pacificus* (Acari: Ixodidae) nymphs in relation to environmental parameters in dense-woodland and woodland-grass habitats. *J Vector Ecol*, *32*, 342-357.

Larsson, C., Comstedt, P., Olsen, B., Bergström, S. (2007). First record of Lyme disease *Borrelia* in the Arctic. *Vector Borne Zoonotic Dis*, *7*, 453-456.

Lees, A. D. & Milne, A. (1951). The seasonal and diurnal activities of individual sheep ticks (*Ixodes ricinus* L.). *Parasitology*, *41*, 189-208.

Lees, A. D. (1946). The water balance in *Ixodes ricinus* L. and certain other species of ticks. *Parasitology*, *37*, 1-20.

Lefcort, H., & Durden, L. A. (1996). The effect of infection with Lyme disease spirochetes (*Borrelia burgdorferi*) on the phototaxis, activity, and questing height of the tick vector

Ixodes scapularis. *Parasitology*, *113*, 97-103.

Lewis, D. (1979). The detection of rickettsia-like microorganisms within the ovaries of female *Ixodes ricinus* ticks. *Parasitol Res*, *59*, 295-298.

Lindgren, E. & Jaenson, T. (2006). Lyme borreliosis in Europe: Influences of climate and climate change, epidemiology, ecology and adaptation measures. Copenhagen: World Health. *WHO Europe*. [Webpage] Available at: http://euro.who.int [Accessed July 7, 2010].

Lindquist, L., & Vapalahti, O. (2008). Tick-borne encephalitis. *Lancet*, *371*, 1861-1871.

Lindstrom, A. & Jaenson, T., 2003. Distribution of the common tick, *Ixodes ricinus* (Acari: Ixodidae), in different vegetation types in Southern Sweden. *J Med Entomol*, 40, 373-378.

Lo, N., Beninati, T., Sassera, D., Bouman, E. A., Santagati, S., Gern, L., et al. (2006). Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick *Ixodes ricinus*. *Environ Microbiol*, *8*, 1280-1287.

Lo, N., Paraskevopoulos, C., Bourtzis, K., O'Neill, S. L., Werren, J. H., Bordenstein, S. R., et al. (2007). Taxonomic status of the intracellular bacterium *Wolbachia pipientis*. *Int J Syst Evol Microbiol*, *57*, 654-657.

LoGiudice, K., Ostfeld, R. S., Schmidt, K. A., Keesing, F. (2003). The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk. *Proc Natl Acad Sci U S A*, 100, 567-571.

Luckhart, S., Mullen, G. R., Durden, L. A., & Wright, J. C. (1992). *Borrelia sp.* in ticks recovered from white-tailed deer in Alabama. *J Wildl Dis*, *28*, 449-452.

Macleod, J. (1932). The bionomics of *Ixodes ricinus* L., the "sheep tick" of Scotland. *Parasitology*, *24*, 382-400.

Maddison, D. R., & Schulz, K.-S. (2007). The Tree of Life Web Project. [Webpage] Retrieved September 6, 2011, from http://tolweb.org

Maetzel, D., Maier, W. A., & Kampen, H. (2005). *Borrelia burgdorferi* infection prevalences in questing *Ixodes ricinus* ticks (Acari: Ixodidae) in urban and suburban Bonn, Western Germany. *Parasitol Res*, *95*, 5-12.

Magalhaes, S., Forbes, M. R., Skoracka, A., Osakabe, M., Chevillon, C., & McCoy, K. D. (2007). Host race formation in the Acari. *Exp Appl Acarol*, *42*, 225-238.

Maiden, M. C. (2006). Multilocus sequence typing of bacteria. *Annu Rev Microbiol*, *60*, 561-588.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., et al. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A*, *95*, 3140-3145.

Mangold, A. J., Bargues, M. D., & Mas-Coma, S. (1998). Mitochondrial 16S rDNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among Metastriata (Acari: Ixodidae). *Parasitol Res*, *84*, 478-484.

Margos, G., Gatewood, A. G., Aanensen, D. M., Hanincova, K., Terekhova, D., Vollmer, S. A., et al. (2008). MLST of housekeeping genes captures geographic population structure and suggests a European origin of *Borrelia burgdorferi*. *Proc Natl Acad Sci U S A*, *105*, 8730-8735.

Margos, G., Vollmer, S. A., Cornet, M., Garnier, M., Fingerle, V., Wilske, B., et al. (2009). A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. *Appl Env Microbiol*, *75*, 5410-5416.

May-Collado, L., & Agnarsson, I. (2006). Cytochrome b and Bayesian inference of whale phylogeny. *Mol Phylogenet Evol*, *38*, 344-354.

McBride, H. M., Neuspiel, M., & Wasiak, S. (2006). Mitochondria: more than just a powerhouse. *Curr Biol*, *16*, R551-60.

McCoy, K. D., Boulinier, T., & Tirard, C. (2005). Comparative host-parasite population structures: disentangling prospecting and dispersal in the black-legged kittiwake *Rissa tridactyla*. *Mol Ecol*, *14*, 2825-2838.

McLain, D. K., Li, J., & Oliver Jr., J. H. (2001). Interspecific and geographical variation in the sequence of rDNA expansion segment D3 of *Ixodes* ticks (Acari: Ixodidae). *Heredity*, *86*, 234-242.

Medlock, J. M., Jameson, L. J., Pietzsch, M. E., & Gilbert, L. (2009). British ticks. *British Wildlife*, *20*, 258-266.

Medlock, J. M., Pietzsch, M. E., Rice, N. V., Jones, L., Kerrod, E., Avenell, D., et al. (2008). Investigation of ecological and environmental determinants for the presence of questing *Ixodes ricinus* (Acari: Ixodidae) on Gower, South Wales. *J Med Entomol*, *45*, 314-325.

Mejlon, H. A., & Jaenson, T. G. T. (1997). Questing behaviour of *Ixodes ricinus* ticks (Acari: Ixodidae). *Exp Appl Acarol*, *21*, 747-754.

Meyer, W., Aanensen, D. M., Boekhout, T., Cogliati, M., Diaz, M. R., & Esposto, M. C. (2009). Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. *Med Mycol*, *47*, 561-570.

Miller, M. P., Bellinger, M. R., Forsman, E. D., & Haig, S. M. (2006). Effects of historical climate change, habitat connectivity, and vicariance on genetic structure and diversity across the range of the red tree vole (*Phenacomys longicaudus*) in the Pacific Northwestern United States. *Mol Ecol*, *15*, 145-159.

Milne, A. (1943). The comparison of sheep tick populations (*Ixodes ricinus* L.). *Annals Appl Biol*, *30*, 240-250.

Milne, A. (1949). The ecology of the sheep tick, *Ixodes ricinu*s L. Host relationships of the tick: Part 1. Review of previous work in Britain. *Parasitology*, *39*, 167.

Milne, A. (1950). The ecology of the sheep tick, *Ixodes ricinus* L., spatial distribution. *Parasitology*, *40*, 35-45.

Mix, A., Bard, E., & Schneider, R. (2001). Environmental processes of the ice age: land, oceans, glaciers (EPILOG). *Quatern Sci Rev, 20*, 627-657.

Moran Cadenas, F., Rais, O., Humair, P. F., Douet, V., Moret, J., & Gern, L. (2007). Identification of host bloodmeal source and *Borrelia burgdorferi* sensu lato in field-collected *Ixodes ricinus* ticks in Chaumont (Switzerland). *J Med Entomol*, *44*, 1109-1117.

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., et al. (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell*, *139*, 1268-1278.

Murrell, A., Campbell, N. J., & Barker, S. C. (1999). Mitochondrial *12S* rDNA indicates that the Rhipicephalinae (Acari: Ixodida) is paraphyletic. *Mol Phylogenet Evol*, *12*, 83-86.

Najm, N.-addeen, Silaghi, C., Bell-sakyi, L., Pfister, K., & Passos, L. M. F. (2011). Detection of bacteria related to *Candidatus* Midichloria mitochondrii in tick cell lines. *Parasitol Res, 110*, 437-442.

Nei, M., & Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York, USA: Oxford University Press.

Norris, D. E., Klompen, J. S., Keirans, J. E., & Black, W. C. IV. (1996). Population genetics of *Ixodes scapularis* (Acari: Ixodidae) based on mitochondrial *16S* and *12S* genes. *J Med Entomol*, *33*, 78-89.

Noureddine, R., Chauvin, A., & Plantard, O. (2011). Lack of genetic structure among Eurasian populations of the tick *Ixodes ricinus* contrasts with marked divergence from North African populations. *Int J Parasitol*, *41*, 183-192.

O'Neill, S. L., Giordano, R., Colbert, A. M., Karr, T. L., & Robertson, H. M. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci U S A*, *89*, 2699-2702.

Ogden, N. H., Lindsay, L. R., Hanincová, K., Barker, I. K., Bigras-Poulin, M., Charron, D. F., et al. (2008). Role of migratory birds in introduction and range expansion of *Ixodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada. *Appl Env Microbiol*, *74*, 1780-1790.

Ogden, N. H., Maarouf, A., Barker, I. K., Bigras-Poulin, M., Lindsay, L. R., Morshed, M. G., et al. (2006). Climate change and the potential for range expansion of the Lyme disease vector *Ixodes scapularis* in Canada. *Int J Parasitol*, *36*, 63-70.

Ogden, N. H., Cripps, P., Davison, C. C., Owen, G., Parry, J. M., Timms, B. J., et al. (2000). The ixodid tick species attaching to domestic dogs and cats in Great Britain and Ireland. *Med Vet Entomol*, 14, 332-338.

Ogden, N. H., Kurtenbach, K. & Nuttall, P. A., 1998. Interstadial and infestation level-dependent variation in the transmission efficiency of *Borrelia burgdorferi* from mice to *Ixodes ricinus* ticks. *Exp Appl Acarol*, 22, 367-372.

Ogden, N. H., Nuttall, P. A. & Randolph, S. E., (1997). Natural Lyme disease cycles maintained via

sheep by co-feeding ticks. *Parasitology*, *115*, 591-599.

Oliver Jr., J. H. (1996). Lyme borreliosis in the southern United States: a review. *J Parasitol*, *82*, 926-935.

Oliver Jr., J. H., Cummins, G. A., & Joiner, M. S. (1993). Immature *Ixodes scapularis* (Acari: Ixodidae) parasitizing lizards from the southeastern U. S. A. *J Parasitol*, *79*, 684-689.

Oliver Jr., J. H., Owsley, M. R., Hutcheson, H. J., James, A. M., Chen, C., Irby, W. S., et al. (1993). Conspecificity of the ticks *Ixodes scapularis* and *I. dammini* (Acari: Ixodidae). *J Med Entomol*, *30*, 54-63.

Olsen, B., Duffy, D. C., Jaenson, T. G., Gylfe, A., Bonnedahl, J., Bergström, S. (1995). Transhemispheric exchange of Lyme disease spirochetes by seabirds. *J Clin Microbiol*, *33*, 3270-3274.

Olsen, B., Jaenson, T. G., Noppa, L., Bunikis, J., & Bergstrom, S. (1993). A Lyme borreliosis cycle in seabirds and *Ixodes uriae* ticks. *Nature*, *362*, 340-342.

Olsen, B., Jaenson, T. G. & Bergstrom, S. (1995). Prevalence of *Borrelia burgdorferi* sensu lato-infected ticks on migrating birds. *Appl Environ Microbiol*, *61*, 3082-3087.

Ozolins, J., & Pilats, V. (1995). Distribution and status of small and medium-sized carnivores in Latvia. *Ann. Zool. Fennici*, *32*, 21-29.

Page, R., & Holmes, E. (1998). *Molecular evolution. A phylogenetic approach.* Oxford, UK: Blackwell.

Pagel Van Zee, J., Geraci, N. S., Guerrero, F. D., Wikel, S. K., Stuart, J. J., Nene, V. M., et al. (2007). Tick genomics: the *Ixodes* genome project and beyond. *Int J Parasitol*, *37*, 1297-1305.

Pal, U., Li, X., Wang, T., Montgomery, R. R., Ramamoorthi, N., M, A., Bao, F., et al. (2004). TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. *Cell*, *119*, 457-468.

Parola, P., & Raoult, D. (2001). Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin Infect Dis*, *32*, 897-928.

Parola, P., Cornet, J.-P., Ose, Y., Miller, R. S., & Thien, H. V. (2003). Detection of *Ehrlichia spp.*, *Anaplasma spp.*, *Rickettsia spp.*, and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. *J Clin Microbiol*, *41*, 1600-1608.

Pichon, B., Egan, D., Rogers, M., & Gray, J. S. (2003). Detection and identification of pathogens and host DNA in unfed host-seeking *Ixodes ricinus* L. (Acari: Ixodidae). *J Med Entomol*, *40*, 723-731.

Pichon, B., Gilot, B., & Pérez-Eid, C. (2000). Detection of spirochaetes of *Borrelia burgdorferi* complexe in the skin of cervids by PCR and culture. *Eur J Epidemiol*, *16*, 869-873.

Pichon, B., Kahl, O., Hammer, B., & Gray, J. S. (2006). Pathogens and host DNA in *Ixodes ricinus* nymphal ticks from a German forest. *Vector Borne Zoonotic Dis*, *6*, 382-387.

Pichon, B., Rogers, M., Egan, D., & Gray, J. S. (2005). Blood-meal analysis for the identification of reservoir hosts of tick-borne pathogens in Ireland. *Vector Borne Zoonotic Dis*, *5*, 172-180.

Piesman, J., & Eisen, L. (2008). Prevention of tick-borne diseases. *Annu Rev Entomol*, *53*, 323-343.

Piesman, J., & Gern, L. (2004). Lyme borreliosis in Europe and North America. *Parasitology*, *129*, S191-S220.

Pietzsch, M. E., Mitchell, R., Jameson, L. J., Morgan, C., Medlock, J. M., Collins, D., et al. (2008). Preliminary evaluation of exotic tick species and exotic pathogens imported on migratory birds into the British Isles. *Vet Parasitol*, *155*, 328-332.

Pietzsch, M. E., Medlock, J. M., Jones, L., Avenell, D., Abbott, J., Harding, P., et al., 2005. Distribution of *Ixodes ricinus* in the British Isles: investigation of historical records. *Med Vet Entomol*, *19*, 306-314.

Putman, R. (2008). Chapter 11: Ungulates, orders Perissodactyla and Artiodactyla. *Mammals of the British Isles: Handbook.* (4th ed., pp. 556-653). Southampton: The Mammal Society.

Qiu, W. G., Bruno, J. F., McCaig, W. D., Xu, Y., Livey, I., Schriefer, M. E., et al. (2008). Wide distribution of a high-virulence *Borrelia burgdorferi* clone in Europe and North America. *Emerg Infect Dis*, *14*, 1097-1104.

Qiu, W. G., Dykhuizen, D. E., Acosta, M. S., & Luft, B. J. (2002). Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the Northeastern United States. *Genetics*, *160*, 833-849.

Randolph, S. E. & Steele, G. M. (1985). An experimental evaluation of conventional control measures against the sheep tick, *Ixodes ricinus* (L.) (Acari : Ixodidae). II. The dynamics of the tick-host interaction. *Bull Ent Res*, *75*, 501-518.

Randolph, S. E. & Storey, K. (1999). Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): implications for parasite transmission. *J Med Entomol*, *36*, 741-748.

Randolph, S. E., Miklisová, D., Lysy, J., Rogers, D. J., Labuda, M. (1999a). Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. *Parasitology*, *118*, 177-186.

Randolph, S. E. (1991). The effect of *Babesia microti* on feeding and survival in its tick vector, *Ixodes trianguliceps*. *Parasitology*, *102*, 9-16.

Regier, J. C., & Shultz, J. W. (1997). Molecular phylogeny of the major arthropod groups indicates polyphyly of crustaceans and a new hypothesis for the origin of hexapods. *Mol Biol Evol*, *14*, 902-913.

Regier, J. C., Shultz, J. W., Zwick, A., Hussey, A., Ball, B., Wetzer, R., et al. (2010). Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. *Nature*, *463*, 1079-1083.

Rich, S. M., Caporale, D. A., Telford III., S. R., Kocher, T. D., Hartl, D. L., & Spielman, A. (1995).

Distribution of the *Ixodes ricinus*-like ticks of eastern North America. *Proc Natl Acad Sci U S A*, *92*, 6284-6288.

Rijpkema, S. G. T., Molkenboer, M. C. H., Schouls, L. M., Jongejan, F., & Schellekens, J. F. P. (1995). Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. *J Clin Microbiol*, *33*, 3091-3095.

Rijpkema, S. G. T., Taxelaar, D., Molkenboer, M. C. H., Noordhoek, G., Plantinga, G., Schouls, L. M., et al. (1997). Detection of *Borrelia afzelii*, *Borelia burgdorferi* sensu stricto, *Borrelia garinii* and group VS116 by PCR in skin biopsies of patients with erythema migrans and acrodermatitis chronica atrophicans. *Clin Microbiol Infect*, *3*, 109-116.

Ros, V. I. D., & Breeuwer, J. A. J. (2009). The effects of, and interactions between, *Cardinium* and *Wolbachia* in the doubly infected spider mite *Bryobia sarothamni*. *Heredity*, *102*, 413-422.

Ruiz-Fons, F., & Gilbert, L. (2010). The role of deer as vehicles to move ticks, *Ixodes ricinus*, between contrasting habitats. *Int J Parasitol*, *40*, 1013-1020.

Ruiz-Fons, F., Fernandez-de-Mera, I. G., Acevedo, P., Hofle, U., Vicente, J., de la Fuente, J., et al. (2006). Ixodid ticks parasitizing Iberian red deer (*Cervus elaphus hispanicus*) and European wild boar (*Sus scrofa*) from Spain: geographical and temporal distribution. *Vet Parasitol*, *140*, 133-142.

Sacchi, L., Bigliardi, E., Corona, S., Beninati, T., Lo, N., & Franceschi, A. (2004). A symbiont of the tick *Ixodes ricinus* invades and consumes mitochondria in a mode similar to that of the parasitic bacterium *Bdellovibrio bacteriovorus*. *Tissue Cell*, *36*, 43-53.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, *4*, 406-425.

Sarih, M., M'Ghirbi, Y., Bouattour, A., Gern, L., Baranton, G., & Postic, D. (2005). Detection and identification of *Ehrlichia spp.* in ticks collected in Tunisia and Morocco. *J Clin Microbiol*, *43*, 1127-1132.

Sassera, D., Beninati, T., Bandi, C., Bouman, E. A., Sacchi, L,. Fabbi, M., et al. (2006). "*Candidatus* Midichloria mitochondrii", an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial lifestyle. *Int J Syst Evol Microbiol*, *56*, 2535-2540.

Sassera, D., Lo, N., Epis, S., Auria, G. D., Montagna, M., Horner, D., et al. (2011). Phylogenomic evidence for the presence of a flagellum and cbb 3 oxidase in the free-living mitochondrial ancestor. *Mol Biol Evol, 28*, 3285-3296.

Scharlemann, J. P., Johnson, P. J., Smith, A. A., Macdonald, D. W., & Randolph, S. E. (2008). Trends in ixodid tick abundance and distribution in Great Britain. *Med Vet Entomol*, *22*, 238-247.

Scott, T. W., Githeko, A. K., Fleisher, A., Harrington, L. C., & Yan, G. (2006). DNA Profiling of human blood in Anophelines from lowlands and highland sites in Western Kenya. *Am J Trop Med Hyg*, *75*, 231-237.

Searle, J. B., Kotlík, P., Rambau, R. V., Marková, S., Herman, J. S., & McDevitt, A. D. (2009). The Celtic fringe of Britain: insights from small mammal phylogeography. *Proc Roy Soc B*, *276*, 4287-94.

Shao, R., & Barker, S. C. (2007). Mitochondrial genomes of parasitic arthropods: implications for studies of population genetics and evolution. *Parasitology*, *134*, 153-167.

Sonenshine, D. (1997). *Biology of Ticks Vol. 1 & 2*. Oxford, UK: Oxford University Press.

Sparagano, O. A. E., & de Luna, C. J. (2008). From population structure to genetically-engineered vectors: New ways to control vector-borne diseases? *Infect Genet Evol*, *8*, 520-525.

Spielman, A., Levine, J. F., & Wilson, M. L. (1984). Vectorial capacity of North American *Ixodes* ticks. *Yale J Biol Med*, *57*, 507-13.

Spielman, A., Clifford, C. M., Piesman, J, & Corwin, M. D. (1979). Human babesiosis on Nantucket Island, USA: description of the vector, *Ixodes* (*Ixodes*) *dammini*, n. sp. (Acarina: Ixodidae). *J Med Entomol*, *15*, 218-234.

Spratt, B. G. (1999). Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. *Curr Opin Microbiol*, *2*, 312-316.

Steele, G. M. & Randolph, S. E. (1985). An experimental evaluation of conventional control measures against the sheep tick, *Ixodes ricinus* (L .) (Acari: Ixodidae). I. A unimodal seasonal activity pattern. *Bull Ent Res*, *75*, 489-499.

Steuber, S., Abdel-rady, A., & Clausen, P.-H. (2005). PCR-RFLP analysis: a promising technique for host species identification of blood meals from tsetse flies (Diptera: Glossinidae). *Parasitol Res*, *97*, 247-254.

Swanson, S. J., Neitzel, D., Reed, K. D., & Belongia, E. A. (2006). Coinfections acquired from ixodes ticks. *Clin Microbiol Rev*, *19*, 708-727.

Tack, W., Madder, M., De Frenne, P., Vanhellemont, M., Gruwez, R., Verheyen, K. (2011). The effects of sampling method and vegetation type on the estimated abundance of *Ixodes ricinus* ticks in forests. *Exp Appl Acarol*, *54*, 285-292.

Talleklint, L., & Jaenson, T. G. (1993). Maintenance by hares of European *Borrelia burgdorferi* in ecosystems without rodents. *J Med Entomol*, *30*, 273-276.

Talleklint, L. & Jaenson, T. G., (1994). Transmission of *Borrelia burgdorferi* s. l. from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden. *J Med Entomol*, *31*, 880-886.

Talleklint, L., & Jaenson, T. G. (1995). Is the small mammal (*Clethrionomys glareolus*) or the tick vector (*Ixodes ricinus*) the primary overwintering reservoir for the Lyme borreliosis spirochete in Sweden? *J Wildl Dis*, *31*, 537-540.

Talleklint, L. & Jaenson, T. G. (1996). Seasonal variations in density of questing *Ixodes ricinus* (Acari: Ixodidae) nymphs and prevalence of infection with *B. burgdorferi* s. l. in south central Sweden. *J Med Entomol*, *33*, 592-597.

Talleklint, L., & Jaenson, T. G. (1997). Infestation of mammals by *Ixodes ricinus* ticks (Acari: Ixodidae) in south-central Sweden. *Exp Appl Acarol*, *21*, 755-771.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony Methods. *Mol Biol Evol 28*, 2731-2739.

Taragel'ova, V., Koci, J., Hanincová, K., Kurtenbach, K., Derdáková, M., Ogden, N. H., Literák, I., et al. (2008). Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe. *Appl Environ Microbiol*, *74*, 1289-1293.

Teixeira, L., Ferreira, A., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol*, *6*, e2.

Telford III., S. R., Mather, T. N., Moore, S. I., Wilson, M. L., & Spielman, A. (1988). Incompetence of deer as reservoirs of the Lyme disease spirochete. *Am J Trop Med Hyg*, *39*, 105-109.

Tobolewski, J., Kaliszewski, M., Colwell, R., & Oliver Jr., J. (1992). Detection and identification of mammalian DNA from the gut of museum specimens of ticks. *J Med Entomol*, *29*, 1049-1051.

Trout, R., Steelman, C., & Szalanski, A. (2009). Population genetics and phylogeography of *Ixodes scapularis* from canines and deer in Arkansas. *Southwestern Entomologist*, *34*, 273-287.

Tzedakis, P. C., Lawson, I. T., Frogley, M. R., Hewitt, G. M., & Preece, R. C. (2002). Buffered tree population changes in a quaternary refugium: evolutionary implications. *Science*, *297*, 2044-2047.

Ullmann, A. J., Lima, C. M., Guerrero, F. D., Piesman, J, & Black, W. C. IV. (2005). Genome size and organization in the blacklegged tick, *Ixodes scapularis* and the Southern cattle tick, *Boophilus microplus*. *Insect Mol Biol*, *14*, 217-222.

Ullmann, A. J., Piesman, J, Dolan, M. C., & Black, W. C. IV. (2003). A preliminary linkage map of the hard tick, *Ixodes scapularis*. *Insect Mol Biol*, *12*, 201-210.

Urwin, R., & Maiden, M. C. (2003). Multi-locus sequence typing: a tool for global epidemiology. *Trends Microbiol*, *11*, 479-487.

van Dam, A. P. V., Kuiper, H., Vos, K., Widjojokusumo, A., De, B. M., Spanjaard, L., et al. (1993). Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. *Clin Infect Dis*, *17*, 708-717.

van Overbeek, L., Gassner, F., van der Plas, C. L., Kastelein, P., Nunes-da Rocha, U., Takken, W. (2008). Diversity of *Ixodes ricinus* tick-associated bacterial communities from different forests. *FEMS Microbiol Ecol, 66*, 72-84.

van Valen, L. (1973). A new evolutionary law. *Evolutionary Theory*, *1*, 1-30.

Vennestrom, J., & Jensen, P. M. (2007). *Ixodes ricinus*: the potential of two-dimensional gel electrophoresis as a tool for studying host-vector-pathogen interactions. *Exp Parasitol*, *115*, 53-58.

Venzal, J. M., Estrada-Pena, A., Portillo, A., Mangold, A. J., Castro, O., de Souza, C. G., et al. (2008). Detection of alpha- and gamma-Proteobacteria in *Amblyomma triste* (Acari: Ixodidae) from Uruguay. *Exp Appl Acarol*, *44*, 49-56.

Vitorino, L. R., Margos, G., Feil, E. J., Collares-Pereira, M., Ze-Ze, L., & Kurtenbach, K. (2008). Fine-scale phylogeographic structure of *Borrelia lusitaniae* revealed by multilocus sequence typing. *PLoS ONE*, *3*, e4002.

Vollmer, S. A., Bormane, A., Dinnis, R. E., Seelig, F., Dobson, A. D. M., Aanensen, D. M., et al. (2011). Host migration impacts on the phylogeography of Lyme borreliosis spirochaete species in Europe. *Env Microbiol*, *13*, 184-192.

Vourc'h, G., Boyard, C. & Barnouin, J. (2008). Mammal and bird species distribution at the woodland-pasture interface in relation to the circulation of ticks and pathogens. *Ann N Y Acad Sci*, *1149*, 322-325.

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., et al. (2011). The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature*, *476*, 450-453.

Wang, G., van Dam, A., Schwartz, I., & Dankert, J. (1999). Molecular Typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. *Clin Microbiol Rev*, *12*, 633-653.

Ward, A. I. (2005). Expanding ranges of wild and feral deer in Great Britain. *Mammal Review*, *35*, 165-173.

Weeks, A. R., Marec, F., & Breeuwer, J. A. J. (2001). A mite species that consists entirely of haploid females. *Science*, *292*, 2479-2482.

Weeks, A. R., Velten, R., & Stouthamer, R. (2003). Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc Royal Soc B*, *270*, 1857-65.

Wernham, C., Toms, M., Marchant, J., Clark, J., Sirowardena, G., & Baillie, S. (2002). *The Migration Atlas: Movements of the Birds of Britain and Ireland.* London, UK: T & AD Poyser.

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol*, *6*, 741-751.

Wesson, D. M., McLain, D. K., Oliver, J. H., Piesman, J, & Collins, F. H. (1993). Investigation of the validity of species status of *Ixodes dammini* (Acari: Ixodidae) using rDNA. *Proc Natl Acad Sci U S A*, *90*, 10221-10225.

Wickramasekara, S., Bunikis, J., Wysocki, V., & Barbour, A. G. (2008). Identification of residual blood proteins in ticks by mass spectrometry proteomics. *Emerg Infect Dis*, *14*, 1273-1275.

Xu, G., Fang, Q. Q., Keirans, J. E., & Durden, L. A. (2003). Molecular phylogenetic analyses indicate that the *Ixodes ricinus* complex is a paraphyletic group. *J Parasitol*, *89*, 452-457.

Zchori-Fein, E., Gottlieb, Y., Kelly, S. E., Brown, J. K., Wilson, J. M., Karr, T. L., et al. (2001). A

newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proc Natl Acad Sci U S A*, *98*, 12555-12560.

Zchori-Fein, E., & Perlman, S. J. (2004a). Distribution of the bacterial symbiont *Cardinium* in arthropods. *Mol Ecol*, *13*, 2009-2016.

Zchori-Fein, E., Perlman, S. J., Kelly, S. E., Katzir, N., & Hunter, M. S. (2004b). Characterization of a "Bacteroidetes" symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of "*Candidatus* Cardinium hertigii." *Int J Syst Evol Microbiol*, *54*, 961-968.

Zink, R. M., & Barrowclough, G. F. (2008). Mitochondrial DNA under siege in avian phylogeography. *Mol Ecol*, *17*, 2107-2121.

Zorenko, T., & Leontyeva, T. (2003). Species diversity and distribution of mammals in Riga. *Acta Zoologica Lituanica*, *13*, 78-86.

Appendix A. List of tick collection sites

Country	Name	Geographic coordinates
UK	Bathampton Woods	51° 23'N, 2° 19'W
	Rainbow Woods	51° 21'N, 2° 20'W
	Inverness	57° 21'N, 2° 20'W
Latvia	Babite	56° 50'N, 23° 48'E
	Kemeri	56° 56'N, 23° 29'E
	Jaunciemis	57° 03'N, 24° 09'E
Portugal	Grandola	38° 17′N, 8°56′W
	Mafra	38° 56′N, 9°20′W
Germany	Bonn	50° 44′N, 7°6′O
	Lennestadt	51° 7′N, 8°4′O
Switzerland	Leukerbad	46° 23'N, 7° 38'E
USA	Mount Riga State Park, Connecticut	42° 4'N, 73° 4'W
	Moore State Park, Massachusetts	42° 19'N, 71° 57'W
	Foothills State Forest, Minnesota	46° 40'N, 94° 41'W
	St Croix State Park, Minnesota	45° 58'N, 92° 35'W
	Black River State Forest, Wisconsin	44° 18′N 90°37′W
	Council Grounds State Park, Wisconsin	45° 11′N 89°44′W
	Bienville National Forest, Mississippi	32° 14'N, 89° 27'W

Appendix B. PCR conditions

Thermocycling conditions for PCR

***I. ricinus* mtMLST**							
Gene	**Initial heating**	**DNA melting**	**Primer annealing**	**Extension**	**Cycles**	**Final extension**	**Expected band size (bp)**
12S	94ºC, 2:00	94ºC, 0:30	46ºC, 1:00	72ºC, 1:30	40	72ºC, 10:00	600
cox1	94ºC, 2:00	94ºC, 1:00	55ºC, 1:00	72ºC, 2:00	35	72ºC, 10:00	785
cox2	94ºC, 2:00	94ºC, 1:00	57ºC, 1:30	72ºC, 2:00	40	72ºC, 10:00	661
cox3	94ºC, 2:00	94ºC, 1:00	45ºC, 1:30	72ºC, 2:00	40	72ºC, 10:00	779
cytb	94ºC, 2:00	94ºC, 0:30	52ºC, 1:30	72ºC, 1:00	40	72ºC, 10:00	780
atp6	94ºC, 2:00	94ºC, 1:00	47ºC, 1:30	72ºC, 2:00	40	72ºC, 10:00	659
***I. ricinus* BMA**							
Vertebrate *12S* (Touch-down)	94ºC, 3:00	94ºC, 0:20	60ºC, 0:30, decrease by 1.0ºC for 8 additional cycles	72ºC, 0:30	9	go to 2nd round below:	
2nd round	-	94ºC, 0:20	52ºC, 0:30	72ºC, 0:30	40	72ºC, 7:00	145
Borrelia							
IGS1	95ºC, 1:00	94ºC, 2:00	52ºC, 0:20	72ºC, 0:45	29	72ºC, 5:00	380
IGS2	95ºC, 1:00	94ºC, 2:00	56ºC, 0:20	72ºC, 0:45	40	72ºC, 5:00	225-270
Wolbachia							
wsp	94ºC, 3:00	94ºC, 1:00	50ºC, 1:00	72ºC, 1:00	40	72ºC, 5:00	600
Cardinium							
16S	94ºC, 4:00	94ºC, 0:40	57ºC, 0:40	72ºC, 0:45	35	72ºC, 5:00	450
gyrB	94ºC, 3:00	94ºC, 1:00	50ºC, 1:00	72ºC, 1:00	40	72ºC, 5:00	600
Midichloria							
16S	94ºC, 2:00	94ºC, 1:00	50ºC, 1:00	72ºC, 2:00	40	72ºC, 10:00	659
adk	94ºC, 2:00	94ºC, 1:00	50ºC, 1:00	72ºC, 2:00	40	72ºC, 10:00	435
lipA	94ºC, 2:00	94ºC, 1:00	50ºC, 1:00	72ºC, 2:00	40	72ºC, 10:00	465
nuoF	94ºC, 2:00	94ºC, 1:00	50ºC, 1:00	72ºC, 2:00	40	72ºC, 10:00	491
ppdK	94ºC, 2:00	94ºC, 1:00	50ºC, 1:00	72ºC, 2:00	40	72ºC, 10:00	501
secY	94ºC, 2:00	94ºC, 1:00	50ºC, 1:00	72ºC, 2:00	40	72ºC, 10:00	484

Primer sequences

Primer name	5'-3' primer sequence
	***I. ricinus* mtMLST**
12S002F	AAAACACTTTCCAGTATTTTTACTTTG
12S601R	GATGATTTGGCTAAACTTGTGC
ATP6004F	AYAAAYYTWTTTTCWATTTTTGATCC
ATP663R	TTAAATTTCRTTWGTRTAWARDGA
COI001F	ATTTTACCGCGATGAYTWTWCTC
COI786R	TCCTGTGRAAACARATRATATGGGA
COII071R	TTTTTCCATGACCATTCAATAATAA
COII731R	ATAAAGTGGTTTAAGAGACCAATGC
COIII001F	ATGATATTYCAYCCWTTTCAYATAG
COIII780R	AWAYTCATCATTATATRAAWGTAAATA
CYTB222F	CCATTCAAATGGAGCATCAA
CYTB1004R	ACAGGGCAAGCTCCTAAGAA
	***I. ricinus* BMA**
12S-6F	CAAACTGGGATTAGATACC
B-12S-9R	5'biotin-AGAACAGGCTCCTCTAG
	Wolbachia
wsp81F	TGGTCCAATAAGTGATGAAGAAAC
wsp691R	AAAAATTAAACGCTACTCCA
	Cardinium
16S CLOf	GCGGTG TAAAATGAGCGTG
16S CLOr1	ACCTMTTCTTAAC TCAAGCCT
	Midichloria
16S12F	CTAGTGGCAGACGGGTGAGT
16S387R	AAGTTAGCCGGGGCTTTTT
adk95F	gcgaaatacttaggaatgaggt
adk605R	aaatcaatcgtgttatctccatca
lipA3F	gatattaggaagtgtctgcac
lipA538R	gctgtagatattgtccaatcg
nuoF118F	cccgatgtagttattgagga
nuoF639R	attatttacagttgttggatgcc
ppdK53F	gtaaatccattctaggaggcaa
ppdK585R	accagcatgttgtaagacga
secY778F	aaagtttatgcaggagattcaac
secY1289R	gtgaggaaataggtttggattc

Appendix C. List of BMA oligo probes

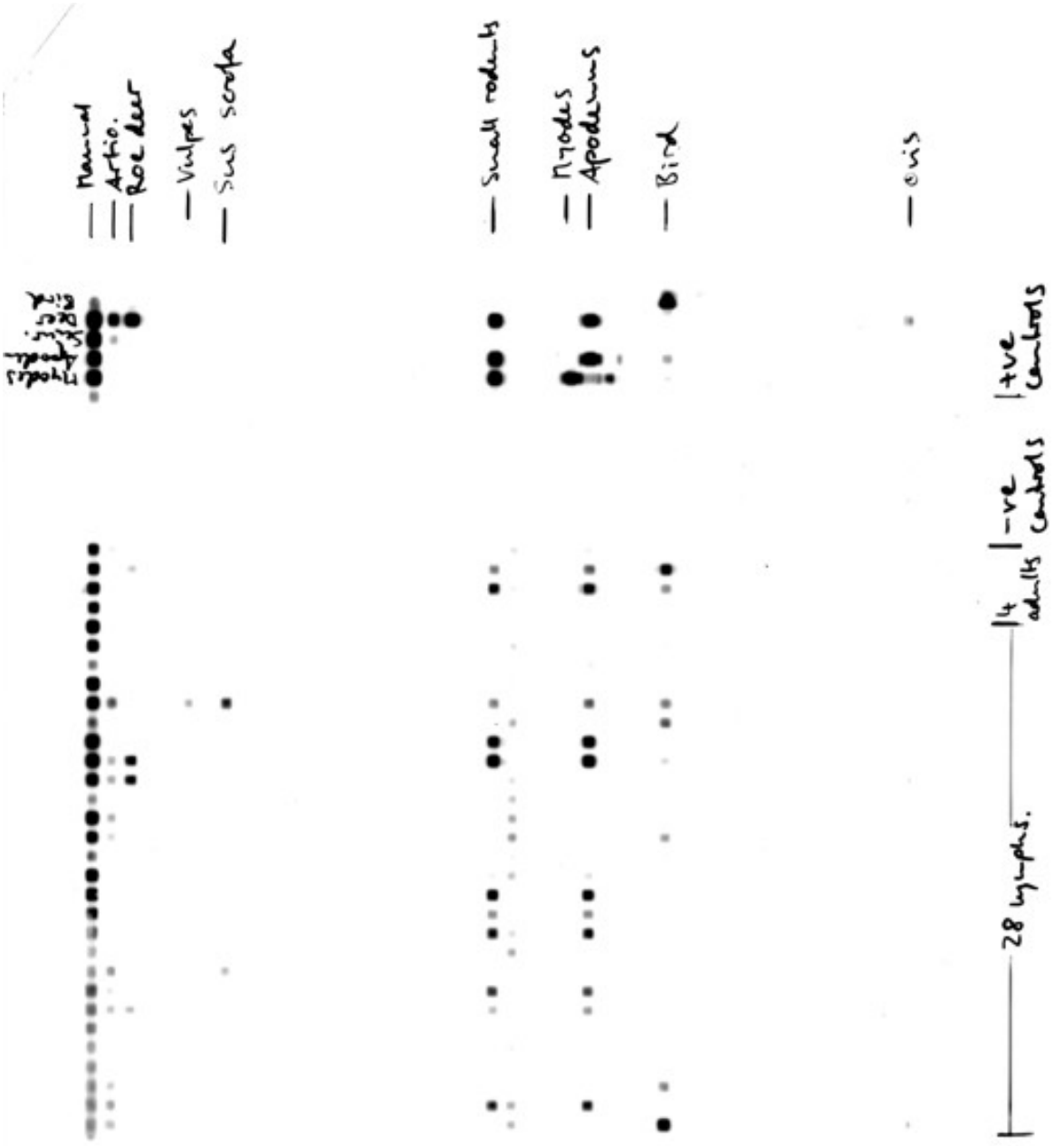
Note: Probes denoted by an asterisk have been newly designed for this study. All others from Humair et al. (2007).

Oligonucleotide probe	Nucleotide sequence (5' - 3')	Target organisms
Mammal	5'amino-AAAACTCAAAGGACTTGGC	Mammalia
Artiodactyl	5'amino-TATTCGCCAGAGTACTAC	Bovidae, Cervidae, Suidae
Roe deer	5'amino-CCTAAACACAAGTAATTAATATAACAA	*Capreolus capreolus*
Red deer*	5'amino-CCT TAA ACA CAA ATA GTT ATG C	*Cervus elaphus*
Fallow deer*	5'amino-CCC TAA ACA CAA ATA GTT GTA	*Dama dama*
Fox	5'amino-CTATAACAAAACAATTCGCCA	*Vulpes vulpes*
Badger	5'amino-GATAACTCACAGAACAAAACT	*Meles meles*
Boar	5'amino-ACCCAAATAGTTACATAACAAAA	*Sus scrofa*
Polecat	5'amino-CTAAACATAAATAATTATCACAACAA	*Mustela putorius*
Stoat	5'amino-CATAAATAGTTCTAACAACAAAAC	*Mustela erminea*
Hare	5'amino-TTAAACCTAAATAATTTCCTAACAAA	*Lepus europaeus*
Rabbit*	5'amino-CTAAACTTTGATAATTTCATAACAAA	*Oryctolagus cuniculus*
Hedgehog	5'amino-GACAGTTACTTAACAAAATTGTA	*Erinaceus europaeus*
Mole	5'amino-ACCAAGACAATCAAGTTAACA	*Talpa europaea*
Neomys	5'amino-ACAAAATTACTCGCCAGAG	*Neomys anomalus, N. fodiens*
N. anomalus	5'amino-TAAACTCAAGTAATTCACAAACA	*Neomys anomalus*
Sorex	5'amino-AATACCCGCCAGAGAAC	*Sorex araneus, S. alpinus*
S. minutus	5'amino-ATCTAACAAGAATACCCGC	*S. minutus, S. coronatus*
S. araneus	5'amino-GGTATTTAACCTAACAAAAATAC	*Sorex araneus*
Squirrel	5'amino-AACATAGACACTCAATTAACAAG	*Sciurus vulgaris*
Dormouse	5'amino-AAACCCTTACTAACGCAAC	*Glis (Myoxus) glis*
Small rodent	5'amino-GGCGGTACTTTATATCCAT	Muroidea (Muridae, Cricetidae)
Mus	5'amino-TGCTTAGCCATAAACCTAAAT	*Mus musculus*

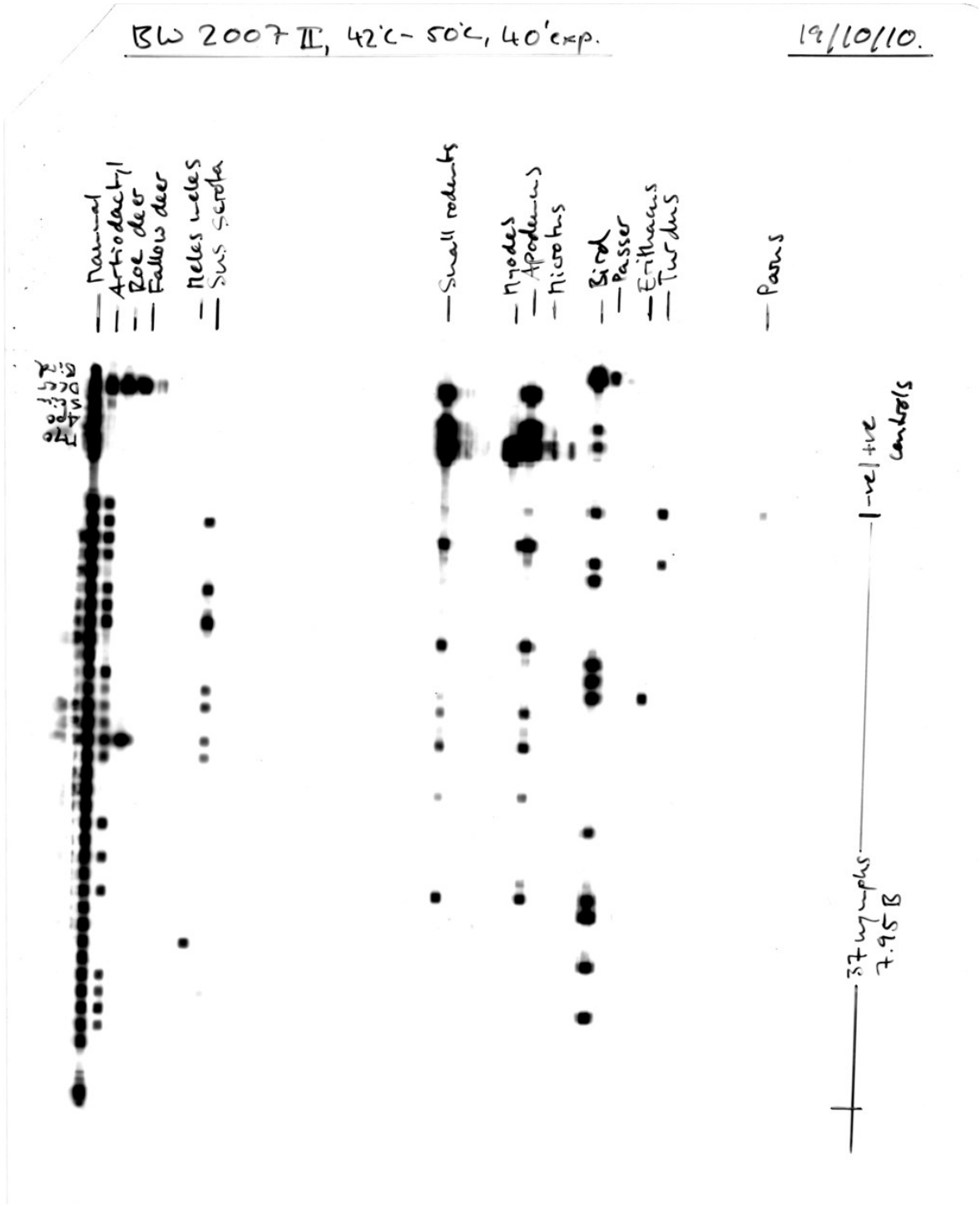
Rattus rattus	5'amino-ACCTTAATAATTACATCTACAAAAAT	*Rattus rattus*
R. norvegicus	5'amino-AACCTTAATAATTAAACCTACAAAAT	*Rattus norvegicus*
Myodes	5'amino-AAACTTCAACATTTATAAAACAAAAG	*Myodes glareolus*
Apodemus	5'amino-TAAACTTAAATAATTTAATAACAAAACTAT	*Apodemus sylvaticus, A. flavicollis*
Micromys/ Microtus	5'amino-CTTAGCCCTAAACTTTAATAATT	*Microtus agrestis, Micromys minutus*
M. arvalis	5'amino-AAACCTCAATAATTTAGAAACAAAAA	*Microtus arvalis*
M. minutus	5'amino-AAACTTTAATAATTCCACAACAAAAT	*Micromys minutus*
Bird	5'amino-TACGAGCACAAACGCTTAA	Aves
Passer*	5'amino-TGATGCTTAAACCTACTAAAG	*Passer domesticus*
Pheasant*	5'amino-AGATGCCTACATACCCAT	*Phasianus colchicus*
Robin	5'amino-ATCTTGATGCTCCACCTTA	*Erithacus rubecula*
Turdus/Parus	5'amino-TGATGCTCGATATTACCTG	*Turdus merula, T. iliacus, T. philomelos, T. pilaris, Parus major, P. caeruleus*
Garrulus	5'amino-TTGACACTCTATGCTACCT	*Garrulus glandarius*
Fringilla/Pyrrhula	5'amino-TGATGCTTACCCCTACTAA	*Fringilla coelebs, F. montifringilla, Pyrrhula pyrrhula*
Prunella	5'amino-TGATGCTTAACCCTACCTA	*Prunella modularis*
Sitta	5'amino-TATACAACCTAAGCATCCG	*Sitta europaea*
Wren	5'amino-TGATGCTTTATATAACCCAAG	*Troglodytes troglodytes*
Parus	5'amino-TGAGCGTCCGCCTGA	*Parus major, P. caeruleus*
Warbler	5'amino-GCTCGATCTTACTGGAG	*Sylvia atricapilla*
Lizard	5'amino-GAGAACTACAAGTGAAAAACT	Lacertidae
Sheep*	5'amino-AATAATTATAAAAACAAAATTATTCGCC	*Ovis aries*

Appendix D. Scanned slides of BMA films

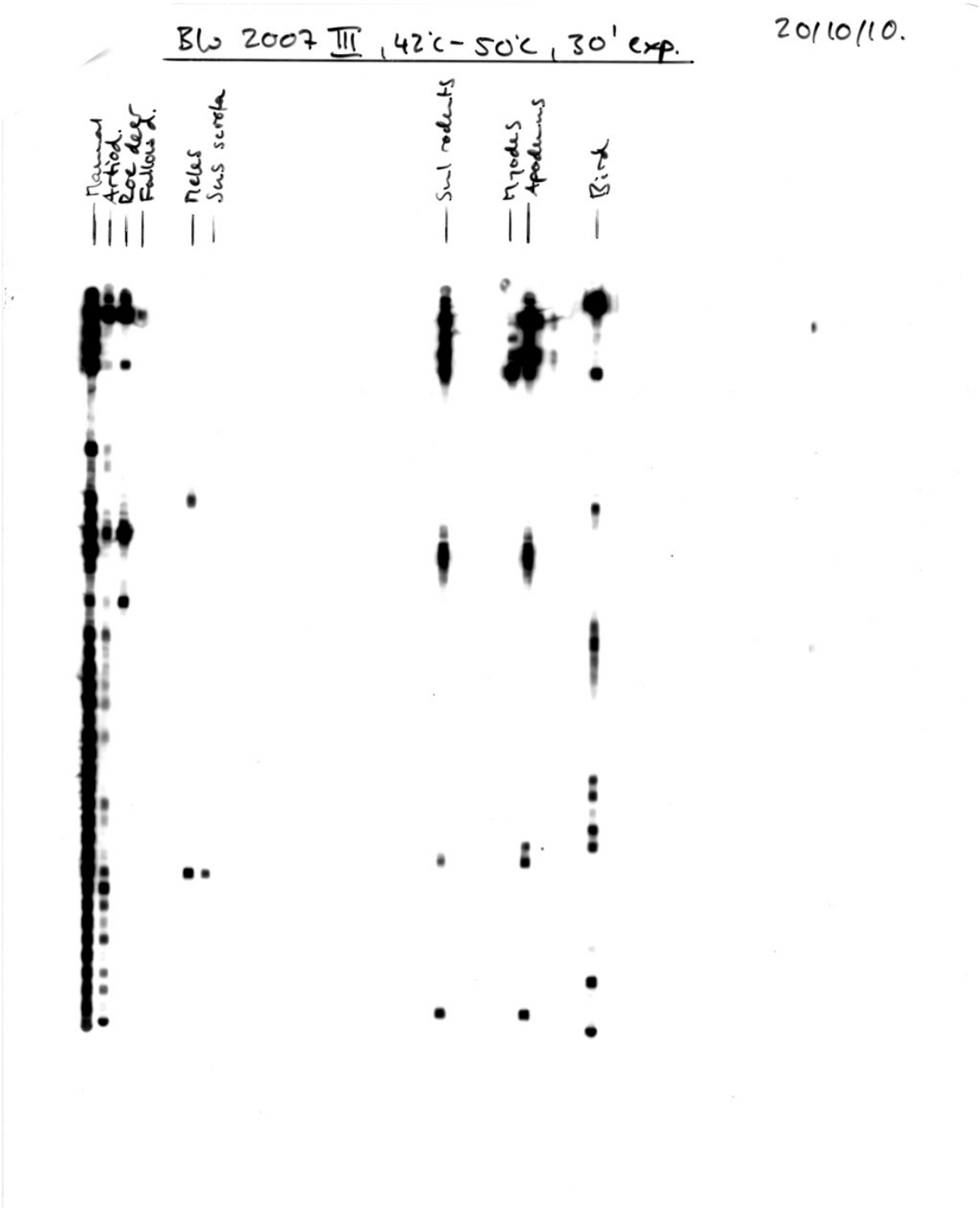
Borrelia negative ticks from BW, Britain, batch I, 2007.



Borrelia negative ticks from BW, Britain, batch II, 2007.



Borrelia negative ticks from BW, Britain, batch III, 2007.



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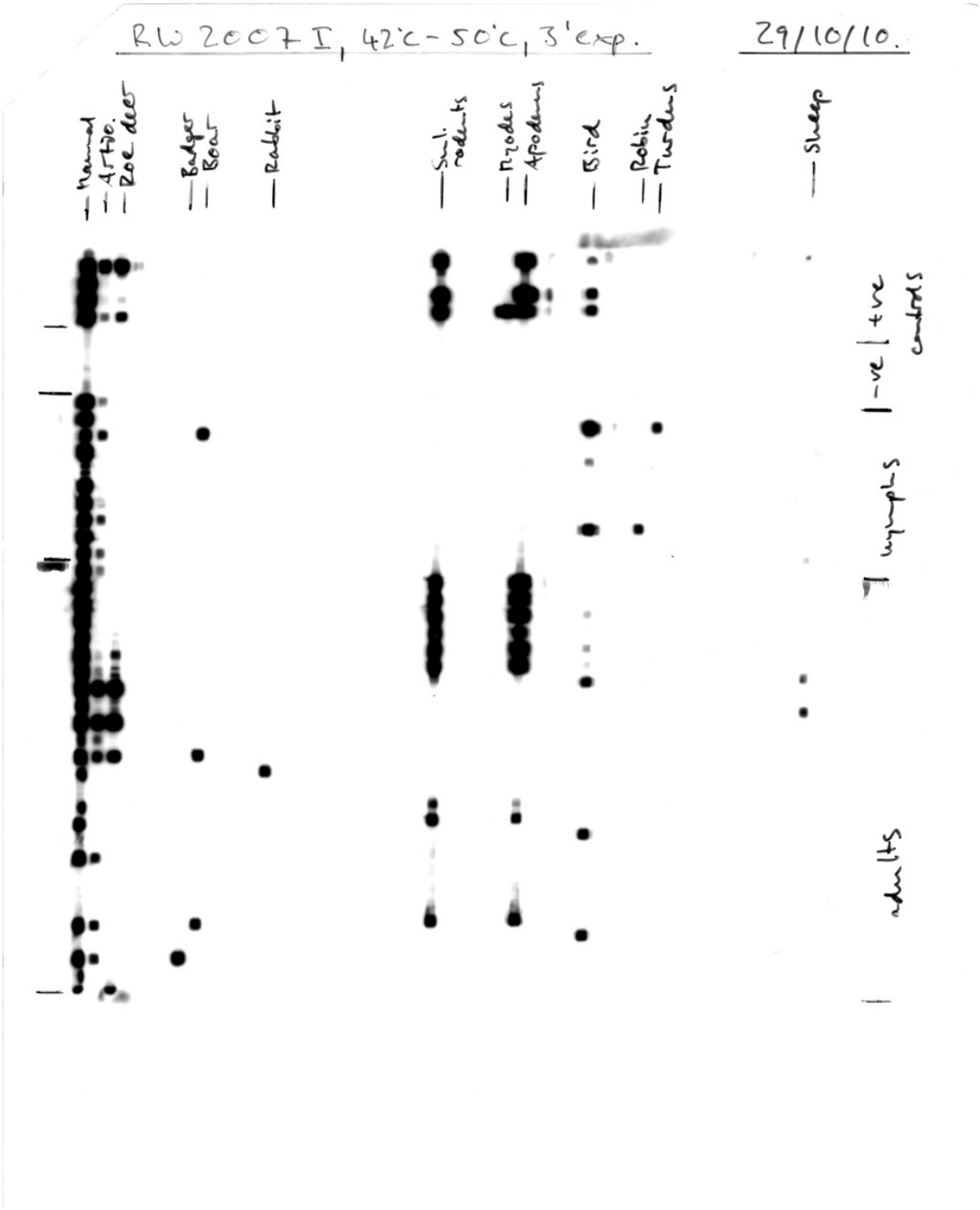

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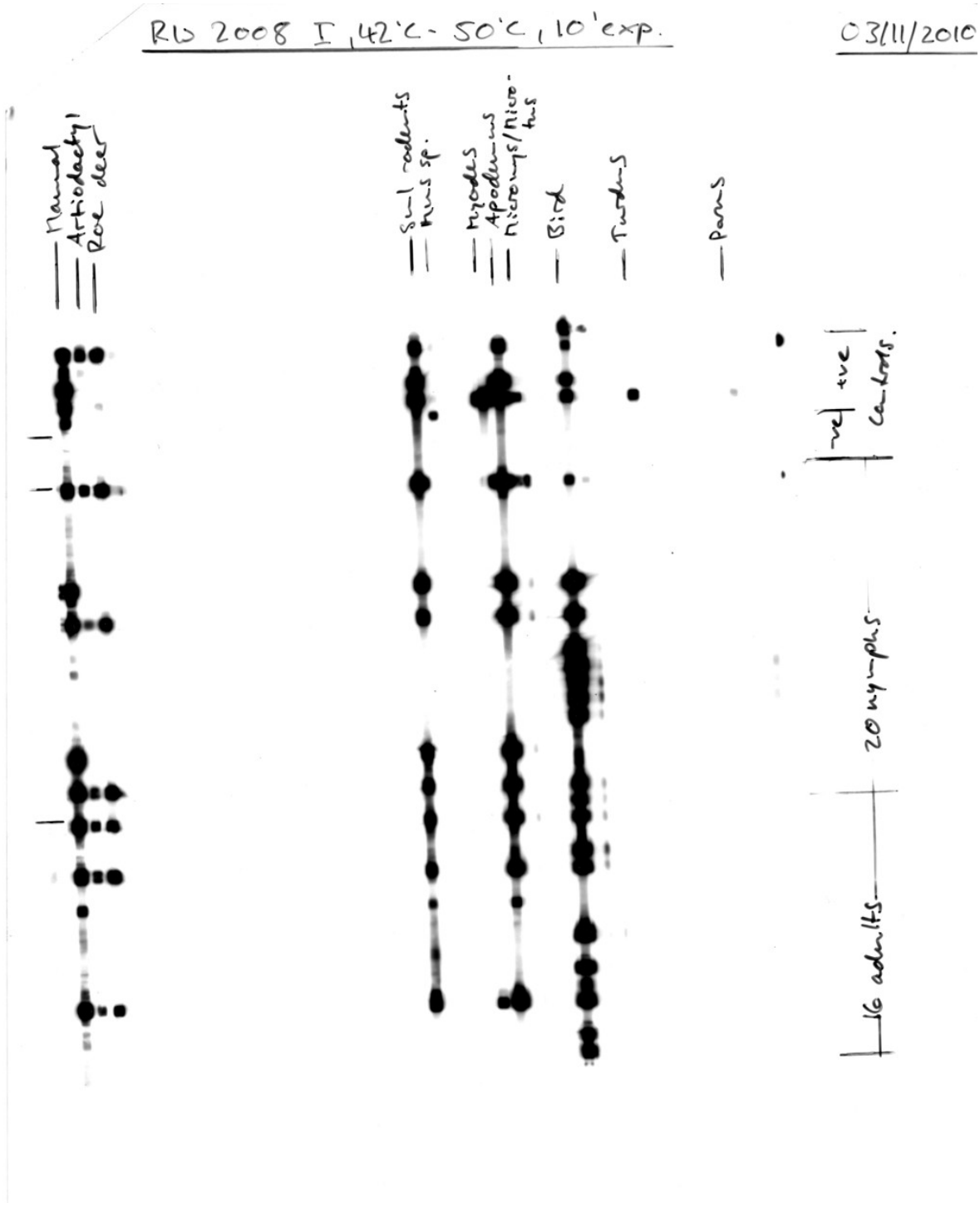

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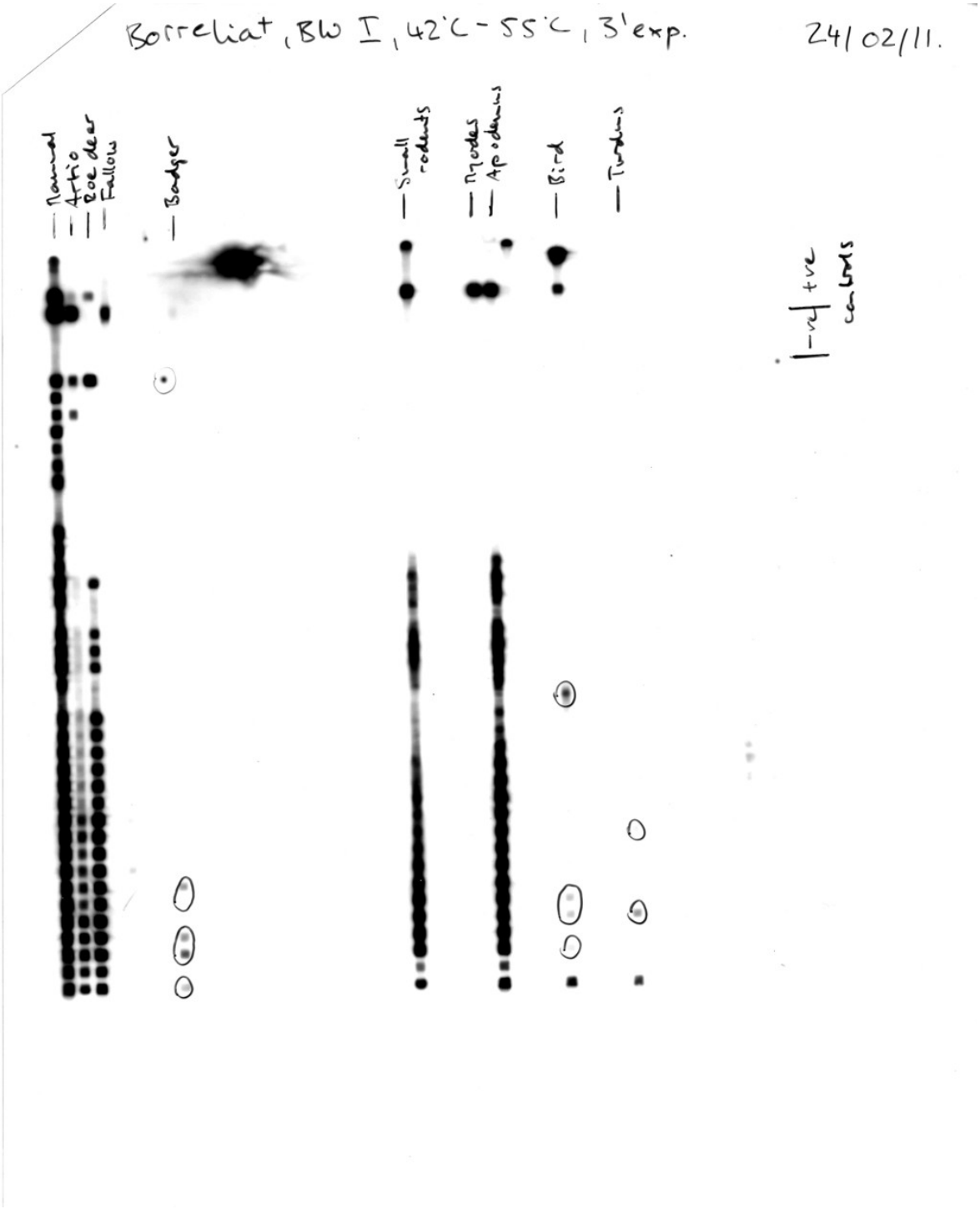

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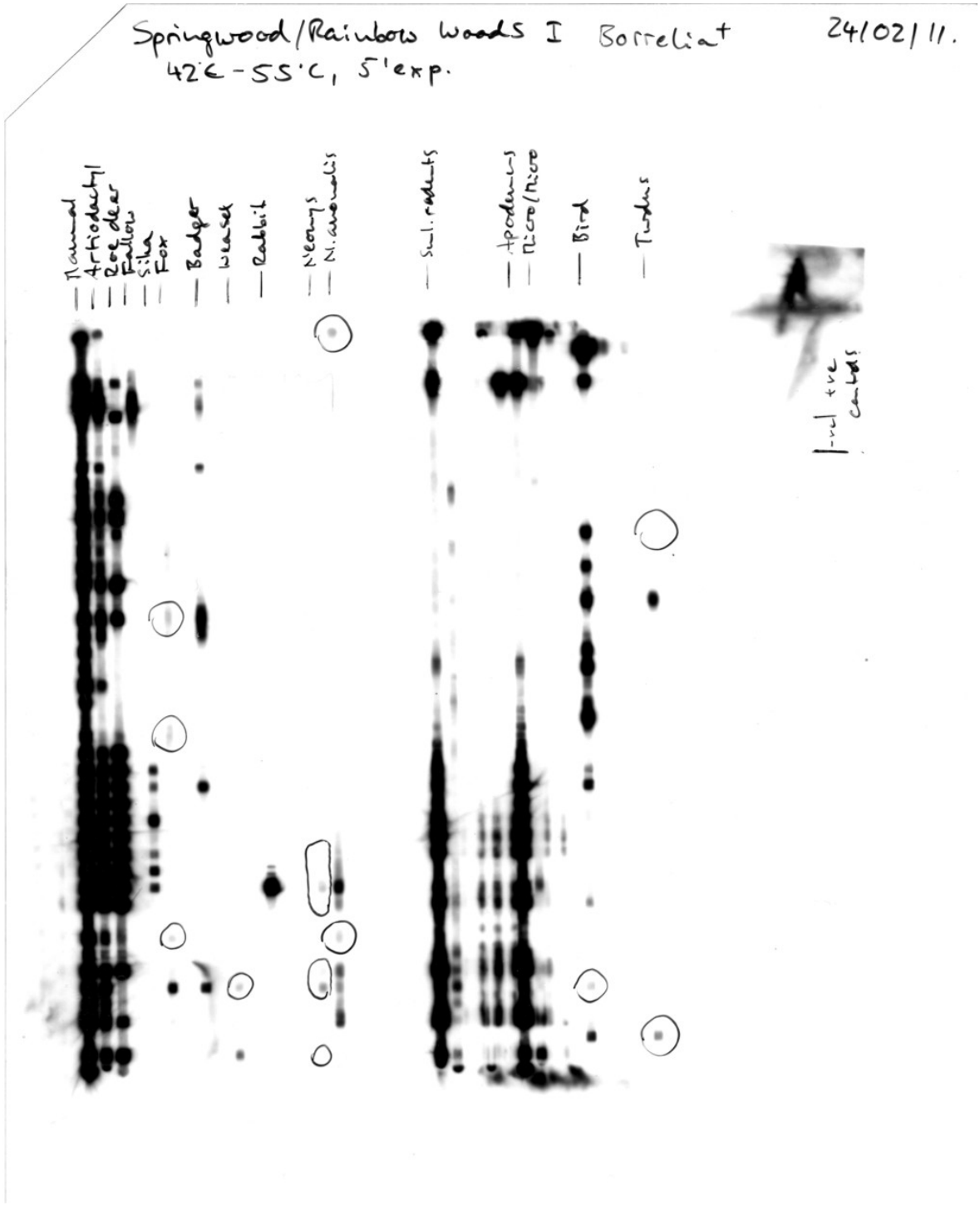
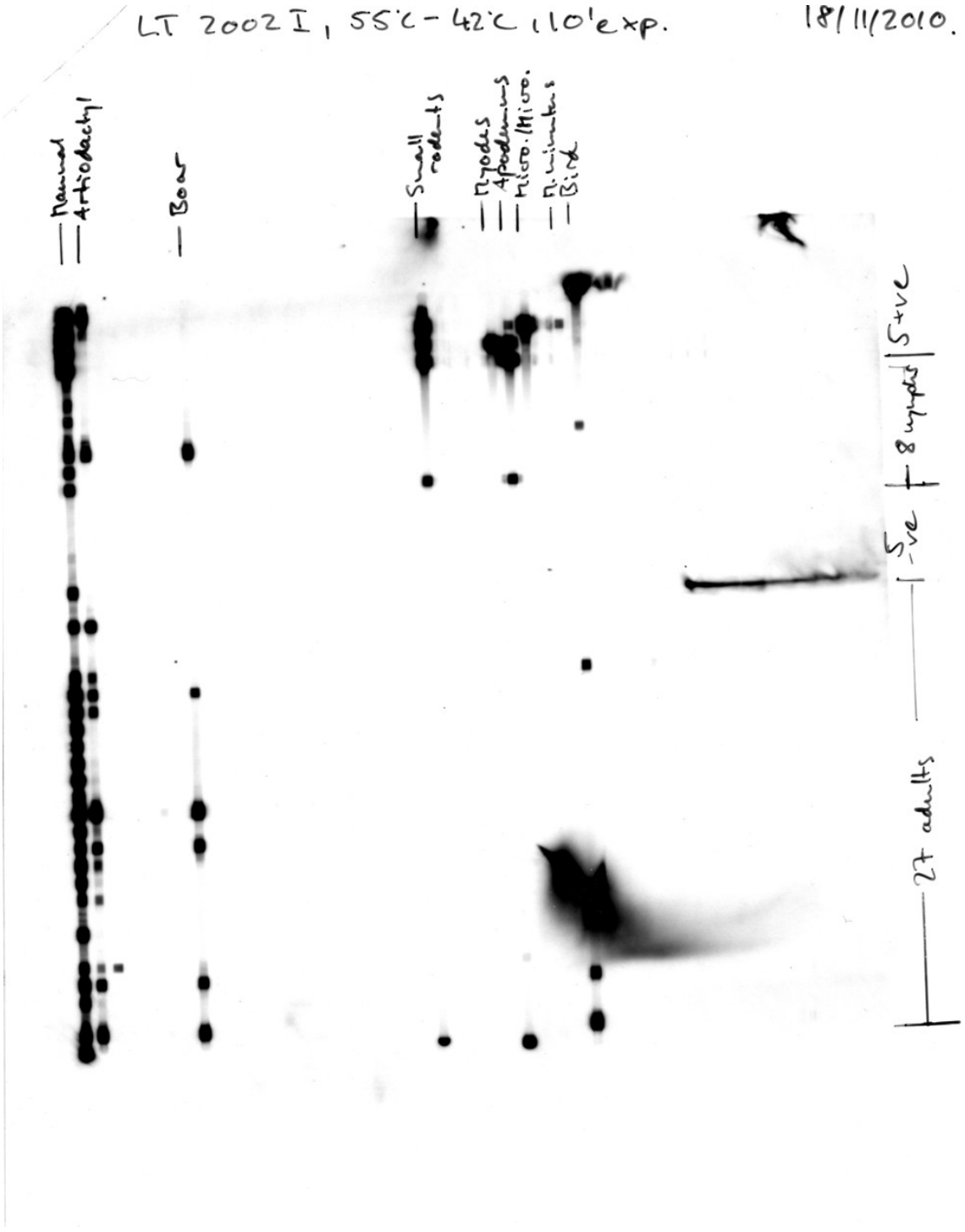


Borrelia negative ticks from Latvia, batch I, 2002.



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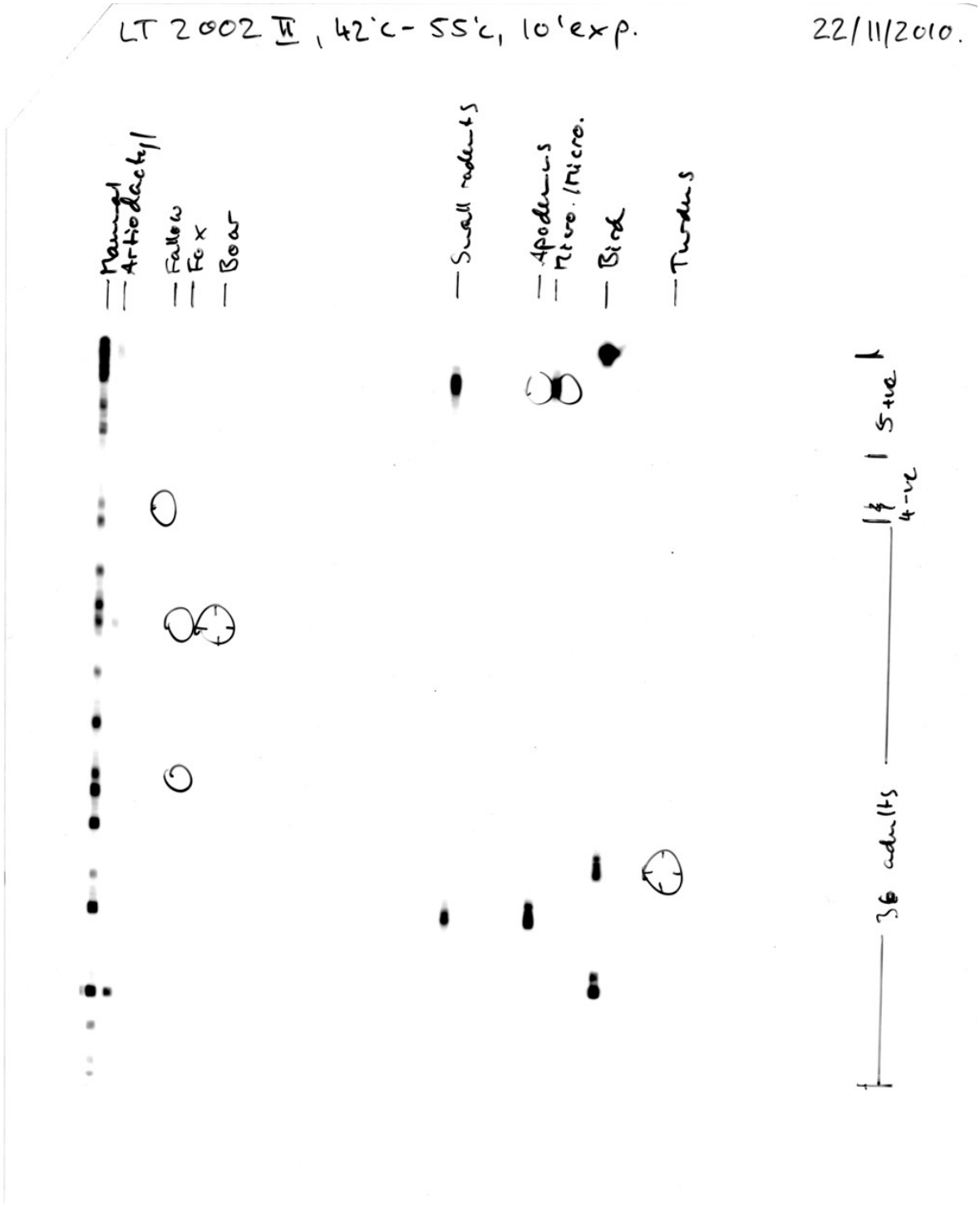

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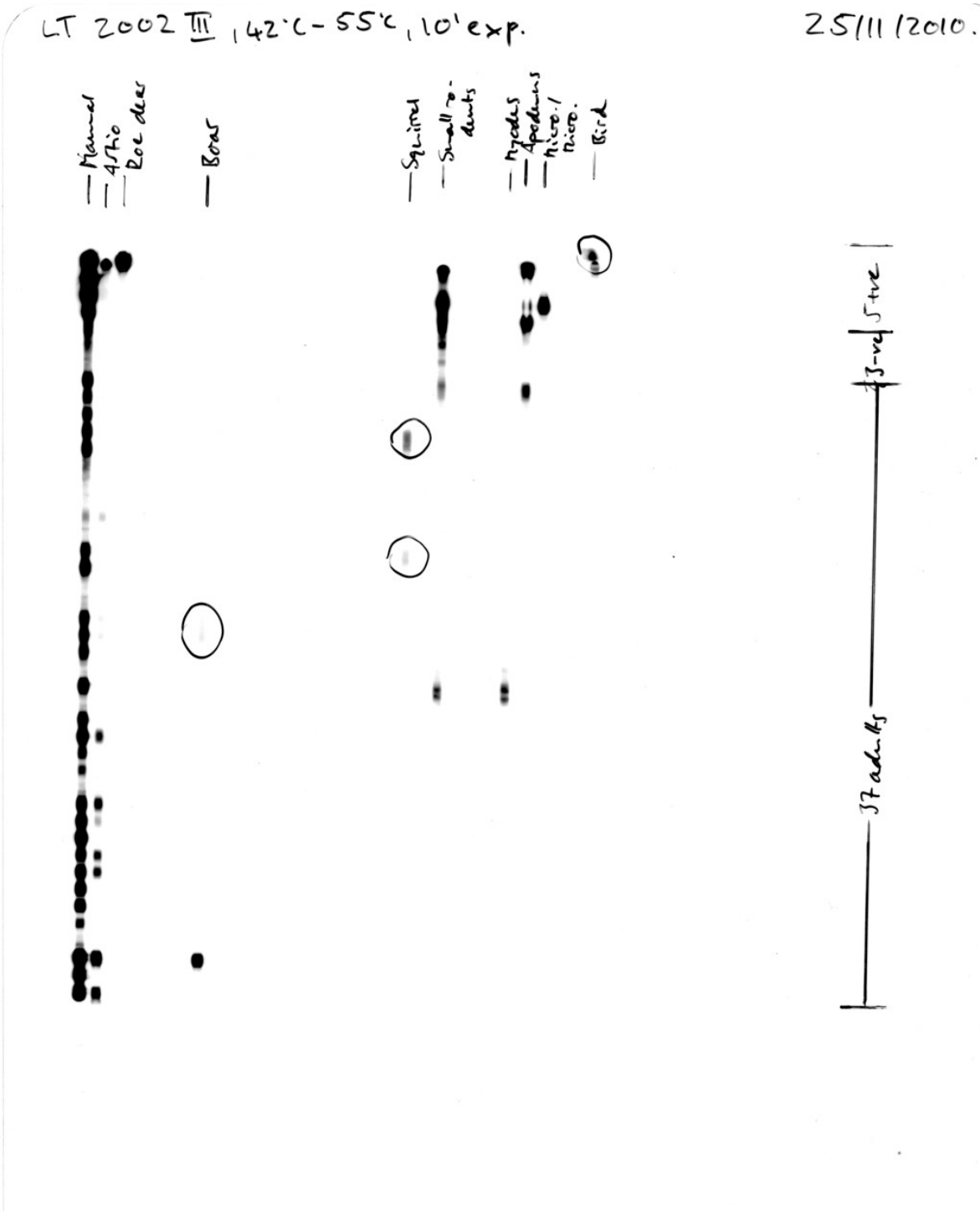
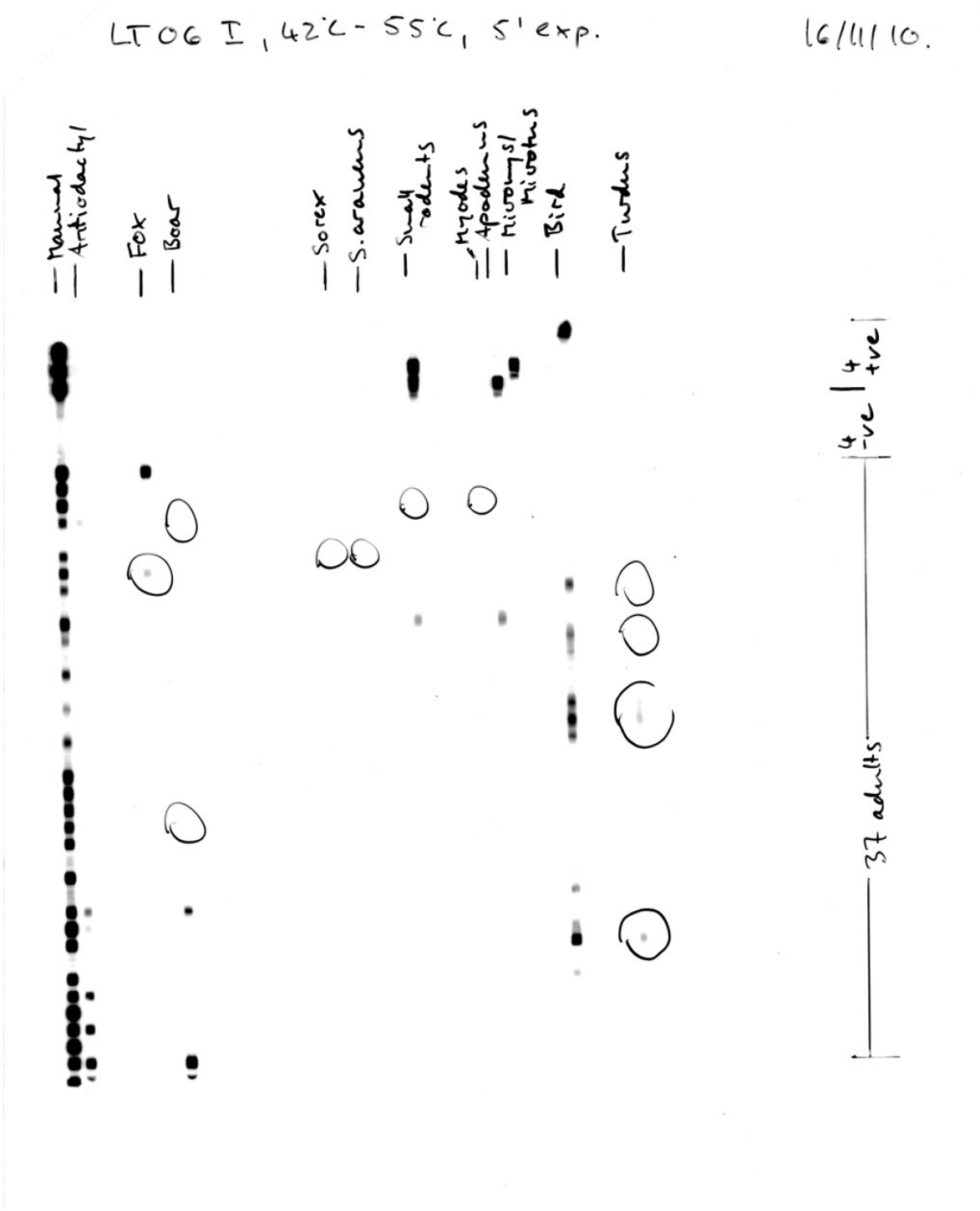
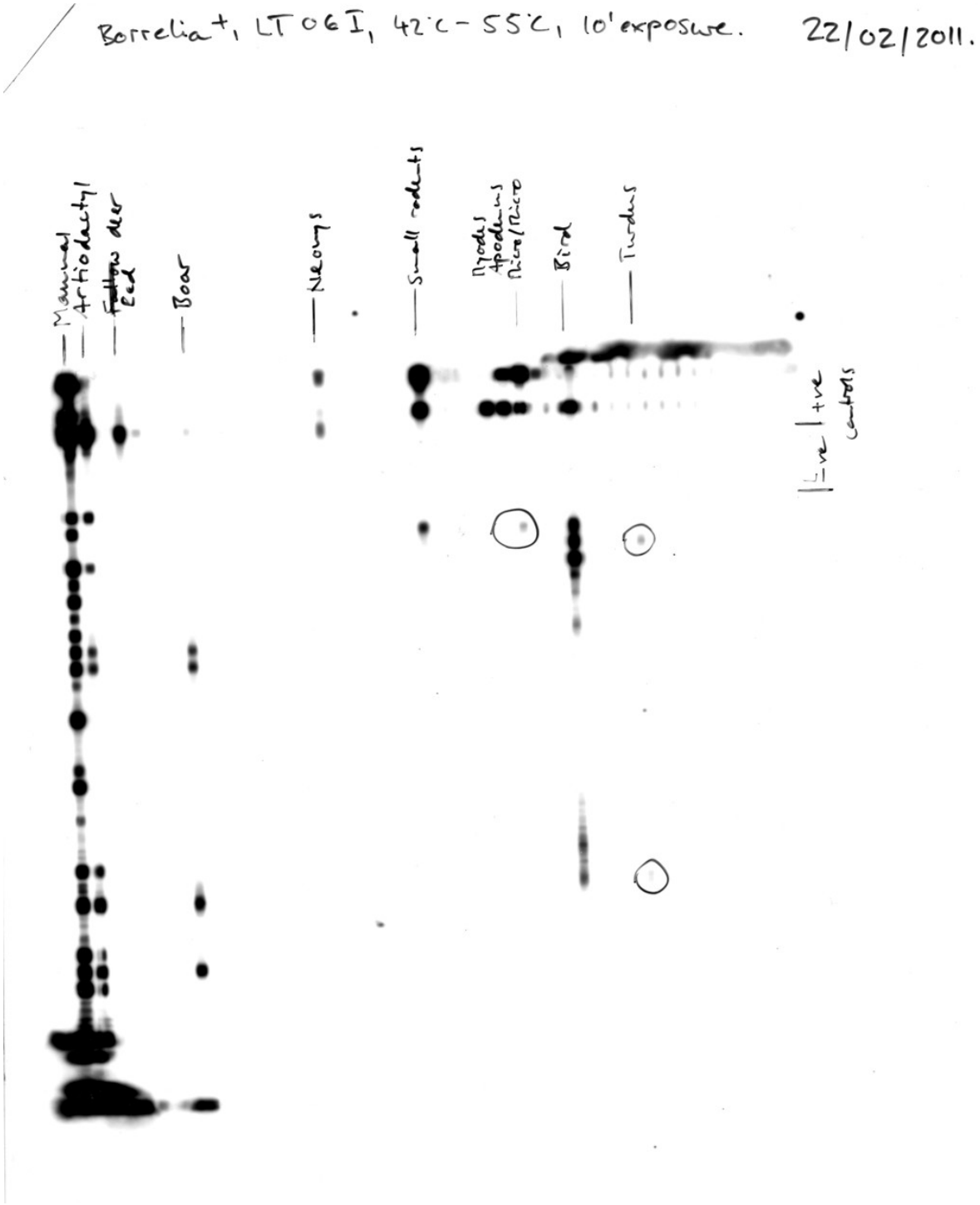


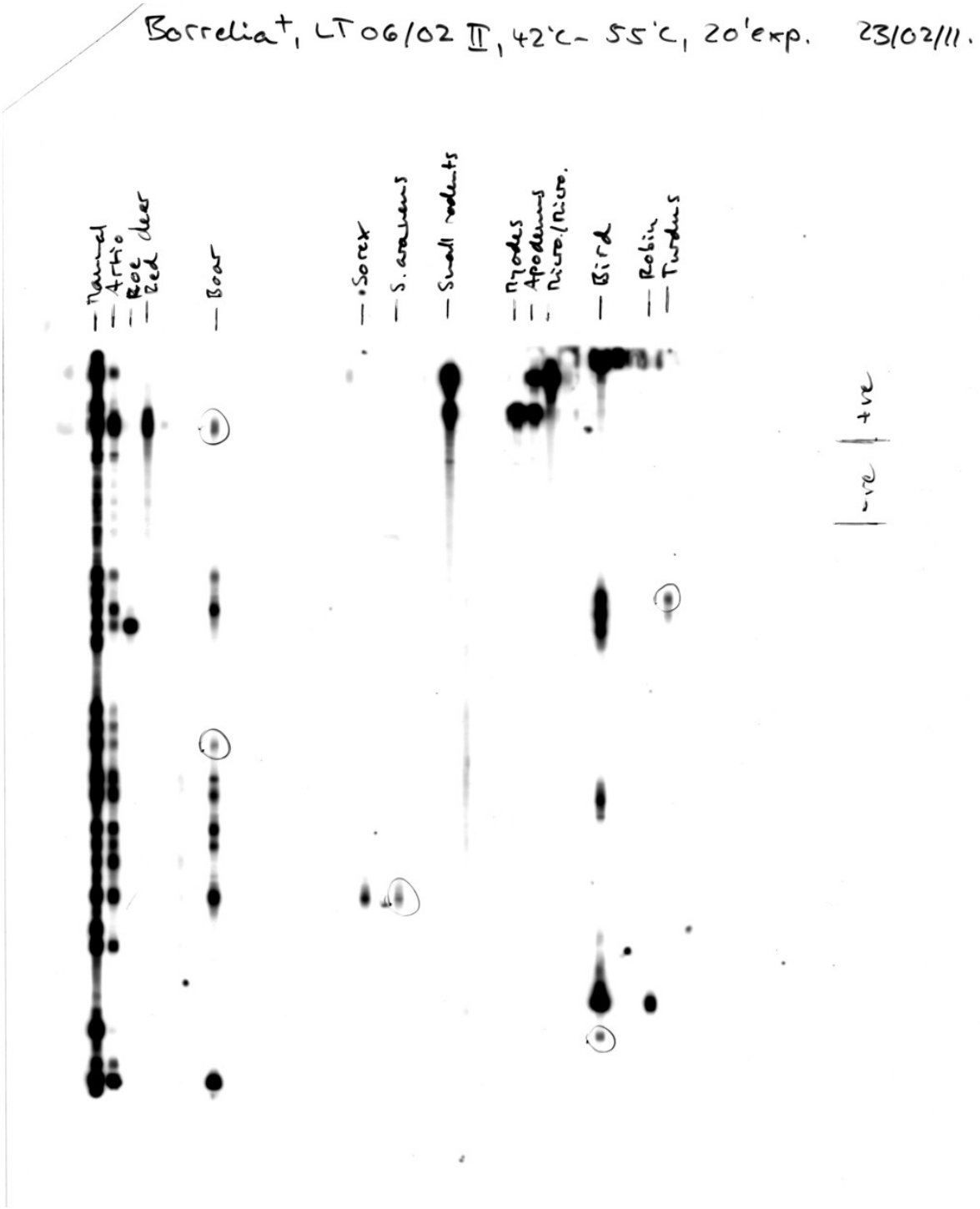
Borrelia negative ticks from Latvia, batch I, 2006.



Borrelia positive ticks from Latvia, batch I, 2006.



Borrelia positive ticks from Latvia, batch II, 2002/2006.



Appendix E. Manuscript of paper

"Multilocus sequence typing reveals geographic population structure of *Ixodes ricinus* ticks using mitochondrial genes"

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Abstract

The hard tick Ixodes ricinus is the principal vector of Lyme borreliosis. This hard tick species transmits a large number of other microbial pathogens that are of importance to animal and human health. The distribution of I. ricinus ranges from Scandinavia to parts of Russia and extends south as far as the Atlas Mountains in North Africa. Here we characterise geographically distinct populations of this important ectoparasite based on multiple mitochondrial genes forming a Multilocus Sequence Typing (MLST) scheme. Internal fragments of approximately 500bp were amplified and sequenced for six protein encoding and ribosomal genes (atp6, coi, coii, coiii, cytB and 12s) in samples of questing nymphs collected in Britain and Latvia in 2006. Although little genetic structure has previously been observed in I. ricinus ticks within Europe, our data clearly differentiate these two populations. We argue that this novel scheme represents a powerful tool for understanding the genetic and geographic structure of I. ricinus ticks, which is important for the monitoring and management of tick-borne diseases.

Introduction

Biogeographical studies, which aim to describe population variation within temporal and spatial frameworks, have been carried out on a wide range of organisms including birds (Morris-Pocock *et al.*, 2008; Mayer *et al.*, 2009), mammals (Hansson & Henttonen, 1988; May-Collado & Agnarsson, 2006), and bacteria (reviewed in Spratt, 1999; Maiden, 2006). Arthropod vectors of disease have received special attention owing to their importance to human and animal health, and these studies have utilised various genetic markers. For example, sandfly species have been studied using rRNA and microsatellites (Aransay *et al.*, 1999; reviewed in Hamarsheh *et al.*, 2009; Parvizi *et al.*, 2010) and mosquito populations have been studied using both of these (Cook *et al.*, 2005) and nuclear genes (Rona *et al.*, 2010).

The hard tick Ixodes ricinus is the principal vector of Lyme borreliosis in Western and Central Europe. This tick species transmits a large number of other microbial pathogens that are of importance to animal and human health. The distribution of I. ricinus ranges from Scandinavia to parts of Russia, and extends south as far as the Atlas Mountains in North Africa. I. ricinus ticks

have previously been typed using 18s rDNA (Mangold *et al.*, 1998), allozymes (Delaye *et al.*, 1997), mitochondrial gene fragments (Xu *et al.*, 2003; Casati *et al.*, 2007) and microsatellites (de Meeus *et al.*, 2002). However, mitochondrial markers have proven their utility due to high mutation rates and low recombination rates (Li, 1997) and have been the primary method of choice in recent years.

Delaye et al. (1997) investigated the population structure of I. ricinus using allozymic data (α-glycero-phosphate-dehydrogenase (α-GPD) and phospho-gluco-mutase (PGM)). They found no evidence of geographic structuring in the ticks, despite differences in disease foci. However, this study was limited to samples from a single country (Switzerland), and used only two polymorphic allozymes. Casati et al. (2007) studied I. ricinus from various European countries using mitochondrial markers (coi, coii, cytB, 12s and mitochondrial control region (CR)), but these authors also reported a lack of geographic structure. In contrast, two studies investigating Ixodes scapularis in the United States reported evidence of geographical structuring. Rich et al. (1995) and Norris et al. (1996) used the 12S and 16S mitochondrial genes to study the I. scapularis populations on the eastern coast of the US. Both studies suggested that two distinct I. scapularis populations exist, one in the Northeast region and one in the Southeast region of the United States.

Other studies have focussed on the closely related species Ixodes uriae, which is a tick specialised to feed on seabirds, but these studies have also yielded contrasting results. Kempf et al. (2009) did not find evidence of geographical structure in this species based on the mitochondrial gene cytochrome oxidase III (coiii), whereas McCoy et al. (2003) did find population differentiation using microsatellite markers. In this latter study, it was argued that the dispersal of ticks was tightly linked to the migration of the host, in this case the black-legged kittiwake (Rissa tridactyla).

The study of bacterial populations has become a key part of understanding the spread of emerging infections and unambiguous genotyping systems have been essential to this process (Gevers *et al.*, 2005). While many different methods have been investigated to monitor the spread of pathogens (reviewed in Maiden, 2006), multilocus sequence typing (MLST) has emerged as an invaluable tool to monitor the spread of virulent bacterial strains or fungal pathogens (Maiden *et al.*, 1998; Enright *et al.*, 2000; Meyer *et al.*, 2009). Advantages are the reproducibility of typing methods between laboratories, portability and data sharing via the internet and more robust analyses than single loci can offer (Maiden *et al.*, 1998; Urwin & Maiden, 2003). There are several aspects that need to be taken into consideration when developing MLST. The genes should be single copy genes, be nearly neutrally evolving and not be prone to recombination. Traditionally, slowly evolving housekeeping genes which are under purifying selection have been chosen (Maiden, 2006).

Mitochondrial genes have all of the required characteristics defined for MLST schemes and have been used successfully as part of a scheme studying Cryptococcus neoformans and Cryptococcus gattii (Meyer *et al.*, 2009), the causative agents of fungal meningitis (Safdieh *et al.*, 2008). We have developed a MLST scheme based on mitochondrial genes to study I. ricinus populations. To increase the phylogenetic resolution of mitochondrial markers, six genes were

chosen for the scheme (ATPase 6, cytochrome oxidase I, II and III, the small rRNA subunit and cytochrome B). The scheme was tested using field collected questing I. ricinus nymphs from two geographically separated regions, i.e. Latvia and Britain. Using phylogenetic and goeBURST analyses we were able to demonstrate that ticks clustered mostly according to their geographic origin. This is the first use of a mitochondrial MLST scheme for an arthropod vector and a novel approach for the molecular discrimination of geographically distinct vector populations.

Results

Ticks samples used in this study

I. ricinus nymphs from Latvia (25 samples) and Britain (25 samples) were used for the comparative analysis (Table 1). In Britain, questing nymphs were collected by blanket dragging in temperate woodland from four locations near Bath, England (Error: Reference source not found) and in temperate woodland from one location near Inverness, Scotland. Tick samples from Latvia were also collected by blanket dragging from three deciduous forest sites surrounding Riga, Latvia (Error: Reference source not found).

Mitochondrial genes statistics

Internal fragments of approximately 450 to 500 bp of six genes (atp6, coi, coii, coiii, 12s and cytB) were used for the MLST scheme. The characteristics of these genes are shown in Table 2.

A dN/dS ratio <1 was shown for each of the six mitochondrial genes indicating that all genes are under purifying selection. The gene coi was the most conserved and 12s the least conserved as shown by frequency of alleles found in the population. Mean nucleotide p-distances (**p**) of concatenated samples from Britain and Latvia were calculated and both samples were similar (0.0039 and 0.0033 respectively).

MLST of Ixodes ricinus

For the MLST analysis, all six mitochondrial genes were sequenced. For each gene, allele numbers were assigned to unique sequences with the first discovered allele given the type number 1, the second 2, and so on. Alleles with identical sequences were given the same allele designation. The allelic profiles were combined in the order of atp6, coi, coii, coiii, 12s and cytB to determine sequence types (ST). The allelic profiles are assigned arbitrary ST numbers and identical combinations of alleles are given identical ST numbers. New allelic profiles are given new ST designations. For the 50 ticks sampled, 43 unique STs were determined (Table 1), 20 for the 25 I. ricinus from Britain, and 25 for the 25 I. ricinus from Latvia. Using these allelic profiles, a goeBURST analysis (Feil *et al.*, 2004; Francisco *et al.*, 2009) (Error: Reference source not found) revealed two main clades (CC-B and CC-L) and three minor clades (CC-1, CC-2 and CC-3). The two major clonal complexes were both almost exclusively composed of samples from one country, with CC-B from Britain and CC-L from Latvia. There were some exceptions, a single tick from Latvia (designated ST428) clustered within CC-B, and a tick from Britain (ST302) was clustered within a Latvian clade (CC-M2). The only other STs which were found to correspond to ticks from both locations were ST106 and ST24, which clustered within the minor groups CC-M1 and CC-L, respectively, which were otherwise composed of Latvian ticks.

Four ticks from the dataset differed from all others by more than three loci and were therefore assigned singletons. None of the singletons were represented by more than one tick. Three STs were represented by two or more ticks and these were all of British origin (ST88, ST144 and ST324) (Error: Reference source not found). ST88 was the most common haplotype in the dataset, corresponding to four ticks from Britain. No pair of Latvian ticks were identical, and each tick corresponded to a unique ST. However, the nucleotide diversity of the Latvian samples was slightly lower than of the British samples (Table 2). ST88 was the predicted founder of the major British clade with most of the other British STs related to this ST in three or more loci.

For each sample, the fragments of the six genes were concatenated, aligned, and used to reconstruct a maximum likelihood tree (Guindon et al., 2005) (Error: Reference source not found). The majority of deep branches in the analysis are well supported with good aLRT values of 75 and over. The phylogeny separated ticks into two major clades, which correspond to the country of origin (Britain or Latvia), although small numbers of ticks from Britain clustered within the Latvian clade and vice versa. These exceptions, most notably ST428 and ST106, were the same as those noted in the goeBURST analysis.

The differentiation between the Latvian and British populations was further confirmed by an F_{ST} value of 0.57732 (p=0.0000), as computed using Arlequin version 3.1 (Excoffier *et al.*, 2005) on the basis of the concatenated sequence. The genetic differentiation between subpopulations calculated the degree of. This value indicates a high level of genetic differentiation (Freeland, 2005), which is consistent with the phylogenetic and MLST analyses. As only two STs were shared between Britain and Latvia, this eludes that the populations constitute distinct lineages with little or no movement of individuals from one population to the other.

Discussion

Here, we have developed a novel system for typing of I. ricinus based on the principals of MLST (Maiden *et al.*, 1998; Enright & Spratt, 1999; Spratt, 1999), using mitochondrial genes to better understand the population structure. I. ricinus ticks from Britain and Latvia were characterised using this scheme and in order to ascertain the degree of population differentiation and geographical clustering. Our data clearly demonstrate that I. ricinus from Britain and Latvia form discrete populations, which seriously challenges the view that the Europe-wide population of I. ricinus is panmictic.

The British and Latvian I. ricinus ticks differ from each other in more than three loci in most cases apart from only two STs, which were noted in both locations. The genetic differentiation of the British and Latvian populations reflects their physical separation, both in terms of geographical distance (approximately 1700km) and physical barriers. It is likely that the English Channel n particular presents a substantial barrier to tick movement. There is evidence that immature tick stages can be carried long distances by migratory or partially migratory birds (Ogden *et al.*, 2008; Pietzsch *et al.*, 2008), and these are probably the host species regularly able to introduce new ticks into Britain. However, the limited number of putative migrants noted in the current study suggests that even when ticks are introduced, they rarely go on to establish new populations. This may be

due to the fact that the survival rate of each tick stage is poor, with 90% of a generation failing to reach the next developmental stage (Randolph et al., 2002). Considering these survival rates, introductions of ticks from the continent to Britain would typically fail, and only in rare circumstances would an introduced tick survive to reproductive age. Randolph et al. (2002) estimate that the survival of only 10 adult ticks would on average require an introduction of 1,000 larvae.

A large number of birds are known to carry I. ricinus ticks, many of whom are migratory passerine birds (Comstedt *et al.*, 2006). One of the most common migrants discussed by Comstedt et al. (2006) is the European robin (Erithacus rubecula). Most of the robins which are hatched in the UK and Ireland spend their lives within a kilometre of their natal site (Wernham *et al.*, 2002). Recovery exercises of this species in the UK from 1990 to 1997 showed 2.3% of the birds captured were from foreign countries. Other species of migrant birds studied in Comstedt et al. (2006) showed low levels of migration to and from the UK, with only 2.8% of the common blackbirds (Turdus merula) captured originating from foreign countries. With the majority of blackbird migration from the UK occurring in October (Wernham *et al.*, 2002), when nymphal number are declining (reviewed in Kurtenbach *et al.*, 2006), the impact of bird migration on the dispersal of I. ricinus may in fact be rather limited.

Furthermore, the likelihood of a successful transmission of a mitochondrial ST to a new region would also be halved as, in the majority of cases, only females are able to impart their genetic material to the next generation (Breton *et al.*, 2007). Bi-parental inheritance of mitochondria is rare in many species, unproven in ticks and unlikely to influence the result significantly (Dimauro & Davidzon, 2005). Therefore, a male tick surviving to reproductive age would not impact on the mitochondrial genetic structure of the future population.

Endosymbiontic bacteria of the genus Wolbachia have been found in both I. scapularis (Benson *et al.*, 2004) and I. ricinus (van Overbeek *et al.*, 2008) at a low prevalence, but as many studies have found no evidence of *Wolbachia* (Niebylski *et al.*, 1997; Noda *et al.*, 1997; Hirunkanokpun *et al.*, 2003), the effects on the population may be negligible. These bacteria may have a profound effect on the sex ratio of tick populations due to reproductive changes that lead to skewed sex ratios by cytoplasmic incompatibility (Stouthamer *et al.*, 1999). Wolbachia infection in tick populations in Europe may lead to one ST sweeping across through the population, but more research is needed to determine the effects if any.

The analysis of nuclear genes could possibly allow for the study of this phenomenon, but high levels of recombination in nuclear genes could pose a challenge to reach meaningful conclusions about the descent of the genes studied.

It is tempting to speculate on when the tick populations in Britain and Latvia diverged. If it is indeed the case that the formation of the English Channel was the main cause, then one might imagine that a single ancestral population existed sometime during the Last Glacial Maximum (LGM, approximately 12,000 years ago), when Britain was connected to mainland (Mix *et al.*, 2001; Searle *et al.*, 2009; Ruiz-Fons & Gilbert, 2010). It is conceivable that deer, which are capable of

carrying large number of ticks (Scharlemann *et al.*, 2008), could then migrate freely between the two regions. According to the level of divergence on our data, this would approximately equate to a mutation rate of ~1.9 per site per million years. However, we note that mutation rates are known to vary widely and further research is required.

In this paper, we show population differentiation based upon the geographical location of two populations of Ixodes ticks in Europe and speculate that this may reflect the physical separation of Britain from Continental Europe. This hypothesis predicts that samples from other countries in Continental Europe should show a lower degree of differentiation to each other than to the British population. Previous studies of I. ricinus have failed to discern differences between populations (Casati et al., 2007), perhaps due to a domination of one gene in the multilocus gene analysis and small sample sizes. The MLST scheme that we have developed uses classical MLST principles that have not been previously applied to I. ricinus. It has provided proof-of-principle data that I. ricinus ticks show population structure, but this needs further investigation using larger sample sizes and samples from different geographic origins. A tool to type Ixodes populations would have exciting applications, as it would enable researchers to study the population dynamics of I. ricinus populations across Europe in a spatial and temporal manner. This scheme may also be able to discriminate tick populations with different ecotypes, e.g. ticks infected with different species of Borrelia, to study the propensity of certain ticks to feed on particular animals.

Methods and Materials

Tick DNA extraction from environmental samples

Total genomic DNA from questing I. ricinus ticks was prepared using the alkaline hydrolysis method previously described (Guy & Stanek, 1991). DNA from ticks that were partially or fully engorged with blood were extracted using ammonia hydrolysis initially. The resulting tick lysate was then used in the Qiagen DNeasy Blood and Tissue extraction kit (Qiagen, Germany) according to the manufacturer's instructions apart from the first two initial steps, in which 200µl of ATL buffer was added to tick lysate. Thirtyµl of proteinase K was added to this mixture and incubated at 56°C for between 12-18 hours. Samples were eluted in the provided elution buffer with two elution steps of 100µl rather than the suggested single 200µl step. Extracted DNA was used directly in PCR applications.

Selection of mitochondrial genes and primer design

Since is no full genome published for I. ricinus, primers could not be designed directly. Using I., I. and I. hexagonus from the I. ricinus-persulcatus group, genes from the mitochondrial genome were used to design primers by aligning these sequences in MEGA version 4.1 and creating primers in conserved regions. These primers are shown in Table 3. Two sets of primers for the amplification and sequencing of 12S were used for PCR and sequencing. Samples in the dataset were initially analysed with the primer pair 12S32F and 12S821R, but further development of primers lead to the pair 12S002F and 12S601R being used with more success with the remaining samples in both PCR and sequencing. The new primers bind to alternative sites to complement the already existing selected region of analysis. The tRNA gene fragments were dismissed, as

400-500bp of sequence data are needed to be available for each gene. Other genes were omitted from further analysis as they were not large enough to accommodate internal primers and to produce large enough legible sequence data from traces, as the first 50bp of trace data is often of poor quality.

PCR

PCR was performed using Bioline Biomix™ master mix at 1X concentration (Bioline, UK). Primers of stock solution of 10pmol were added to the PCR reaction according to the conditions shown in Table 4. Template DNA (2µl) was added to PCR reactions. Volumes were increased to 5µl if PCR reactions failed and required repeating. Reaction volumes were adjusted to 25µl with sterile distilled water. PCR samples were heated according to a variety of thermal cycles depending on the properties of the PCR and optimisation criteria as detailed in Table 4.

Extraction of DNA from agarose gel slices

A minority of PCR reactions produced multiple bands and therefore could not be used for sequencing directly. Other reactions produced poor products so bands were cut out and purified to decrease the probability of residual DNA fragments interfering with the sequencing reaction. Using a UV-transillu-minator, PCR products were visualised in agarose gels and cut out of the agarose gel using a sterile straight edged razor blade for each band in each PCR product range. DNA was extracted from gel slices using a Qiagen QIAquick kit according to the manufacturer's instructions, including all optional steps for maximum purity of samples (Qiagen, Germany).

DNA sequencing

Forward and reverse nucleotide sequences of PCR amplicons were sequenced by Qiagen Genomics, Germany, and Agencourt, USA, using PCR primers (Table 3).

MLST scheme

Forward and reverse sequences were analysed using DNASTAR Lasergene SeqMan (version 7.1.0). DNA traces were aligned and trimmed according to the selected allele type region. They were error checked by using automatic ambiguous base matching functions and by manually assessing poor quality bases.

Alleles were assigned to sequences using a non-redundant data base (NRDB) (Gish, 2008). New allele types were verified by aligning each new sequence with all existing allele types of that gene in MEGA version 4.1 (Tamura et al., 2007). Differing bases were verified by examining the original traces. New alleles were assigned if no other alleles matched the new sequence.

After allocation of sequence types according to the allelic profile, singleton sequence types were compared with the entire set of sequence types to find the closest match. New allele types, which differed from the more common sequence types, were reanalysed and differing bases checked once again in the sequence traces to further confirm the sequence identity.

Gene statistics

Values for the overall non-synonymous and synonymous substitutions (dN/dS) of concatenated sequences were determined in MEGA version 4.1 (Tamura *et al.*, 2007), using the modified Nei-Gojobori method and the p-distance model. All positions containing alignment gaps and missing

data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Coding and non-coding domains were assigned. All sites were included.

Nucleotide distances were calculated for all samples and for British and Latvian samples independently. Coding and non-coding domains were assigned and distances were determined in MEGA version 4.1. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). All sites were included.

Sequence alignment

Verified sequences were aligned in MEGA version 4.1 using the default setting for ClustalW alignments. After alignment, some minor editing by hand was used for the adjustment of gaps that were created during the alignment process.

Model testing

Alignments of sequences were tested for suitable models in FindModel (Tao et al., 2009). FindModel incorporated several processes to determine the correct model for the submitted data. Weighbour trees (Bruno et al., 2000) based on Jukes-Cantor distances were used as starting trees. PAML (Yang, 1997, 2007) was used to calculate likelihood. AIC scores were calculated using MODELTEST (Posada & Crandall, 1998).

Maximum likelihood trees

Maximum likelihood trees were constructed using PhyML 3.0 (Guindon & Gascuel, 2003), hosted in the ATGC Montpellier bioinformatics platform. The substitution model was determined in MODELTEST as previously described for each dataset. Starting trees were set as BIONJ, with the tree improvement settings of Sub-tree Pruning and Regrafting (SPR) and Nearest Neighbour Interchange (NNI) with appropriate Likelihood Ratio Test (aLRT) SH (Shimodaira-Hasegawa)-like branch support parameters. All other parameters were set as default values. Open slashes in branches indicate that the branch is not to scale.

goeBURST

The program goeBURST version 1.2.1 (Francisco et al., 2009) is downloadable from the internet. Datasets were constructed using allelic profiles and STs and were clustered according to BURST rules such as those implemented in eBURST (Feil et al., 2004). Samples with the same STs form nodes, which are proportionally sized to reflect the frequency of the ST within the population i.e. larger nodes, indicate STs that are more common. Nodes are related to each other with links. These links are of various colours and are determined as follows: Black solid – single locus variant drawn without recourse to tiebreak rules, Black dotted – Triple locus variant, Black dashed – single locus variant drawn using tiebreak rule 2, grey dashed – single locus variant drawn using tiebreak rule 1 and grey solid – double locus variant. More information on these clustering rules can be found in Francisco et al. (2009). Nodes differing from other nodes by more than three alleles are called singletons and are not linked to other nodes.

Arlequin statistical analyses

The program Arlequin version 3.1 (Excoffier et al., 2005) is downloadable from the internet (http://cmpg.unibe.ch/software/arlequin3). Populations were created using the grouping function

according to the country of origin or other appropriate factor described for individual analyses. F_{ST} values were computed at significance level of 0.05 with 10,000 permutations. Values of zero for F_{ST} indicate that the populations are completely homogenous, values at one indicate disparate populations, and values greater than 0.25 indicate a large amount of genetic diversity (Freeland, 2005).